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LIPID RAFT-BASED STRATEGIES TO INTERFERE WITH HIV-1 INFECTION

TESIS DOCTORAL

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Dedicated to Filipe

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LIST OF ABBREVIATIONS

6HB: Six-helix bundle.
ADA-GT11: ADA HIV-pseudotypes produced in GT11-treated cells.
ADA-T20: ADA HIV-pseudotypes produced in T20-expressing cells.
AIDS: Acquired immunodeficiency syndrome.
APC: Antigen-presenting cell.
APOBEC3G: Apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3G.
BSA: Bovine serum albumin.
Ch: Cholesterol.
CTx: Cholera toxin.
D: Diffusion coefficient.
DAPI: 4',6-diamidino-2-phenylindole.
DC-SIGN: Dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin.
DEPE: Dielaidoylphosphatidylethanolamine.
DHCDase: Dihydroceramide desaturase.
dhSM: Dihydrosphingomyelin.
DIG: Detergent-insoluble glycolipid-enriched membrane.
DMEM: Dulbecco's modified essential medium.
DNA: Deoxyribonucleic acid.
DRM: Detergent-resistant membrane.
EDTA: Thylenediaminetetraacetic acid.
EGF: Epidermal growth factor.
ELISA: Enzyme-linked immunosorbent assay.
Env: Envelope.
FCS: Fetal calf serum.
FITC: Fluorescein isothiocyanate.
FP: Fusion peptide.
FRET: Fluorescence resonance energy transfer.
G418: Geneticin.
GalCer: Galactosyl ceramide.
GFP: Green fluorescent protein.
Gp: Generalised polarization.
GPCR: G-protein coupled receptor.
GPI: Glicosylphosphatidylinositol.
GUV: Giant unilamellar vesicle.
HAART: Highly active antiretroviral therapy.
HIV-1: Human immunodeficiency virus type 1.
HIV-2: Human immunodeficiency virus type 2.
HPLC-MS: High performance liquid chromatography coupled to time of fly mass spectrometry.
HR1: Heptad repeat 1.
HR2: Heptad repeat 2.

IBEC: Institute for Bioengineering of Catalonia.
IC_{50}: Inhibitory concentration (50%).
IC_{90}: Inhibitory concentration (90%).
IFNγ: Interferon gamma.
IIQAB: Chemical and environmental research institute of Barcelona.
IL-2: Interleukin 2.
LAT: Linker of activation of T cell.
L_d: Liquid-disordered.
LD_{50}: Lethal dosis (50%).
LDLR: Low-density lipoprotein receptor.
L_o: Liquid-ordered.
LTR: Long terminal repeat.
mAb: Monoclonal antibody.
MHC: Major histocompatibility complex.
MIP-1α: Macrophage inflammatory protein-1 alpha.
MIP-1β: Macrophage inflammatory protein-1 beta.
mRNA: Messenger ribonucleic acid.
MTS: Soluble tetrazolium salt.
NF-κβ: Nuclear factor kappa beta.
NIH: National institutes of health.
NL4.3-T20: NL4.3 HIV-pseudotype produced in T20-expressing cells.
P56Lck: Lymphocyte-specific protein tyrosine kinase.
PBMC: Peripheral blood mononuclear cell.
PC: Phosphorylcholine.
PE: Phosphoroethanolamine.
PHA-L: Phytohemmaglutinin-L.
RLU: Relative light unit.
RNA: Ribonucleic acid.
RPMI: Roswell Park Memorial Institute medium.
SDF-α: Stromal-derived cell factor-1 alpha.
SDS-PAGE: Sodium dodecyl sulfate polyacrylamide gel electrophoresis.
SHIV: Simian human immunodeficiency virus.
SIV: Simian immunodeficiency virus.
SLP76: SH2-domain-containing leukocyte protein of 76 KDa.
SM: Sphingomyelin.
SP: Signal peptide.
SPT: Single-particle tracking.
TCR: T cell receptor.
TIRFM: Total internal reflection fluorescence microscopy.
T_m: Melting temperature.
UPV/EHU: Basque Country university.

VSVG: Vesicular stomatitis virus glycoprotein.
ZAP70: Zeta chain-associated protein kinase 70.

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SUMMARY

Human immunodeficiency virus type 1 (HIV-1) infection entails the sequential interaction of the viral Env complex with the cellular CD4 receptor and a chemokine coreceptor, culminating in the fusion of the viral and cellular membranes. Sequestration of membrane cholesterol and the inhibition of glycosphingolipid synthesis have suggested a role of liquid ordered (l_o) raft microdomains in HIV-1 entry into target cells.

We devised two different strategies to inhibit HIV-1 infection by targeting lipid rafts in the host cell. First, we targeted a fusion inhibitor (T20) to the membrane of target cells, specifically inside or outside lipid rafts. Notably, we found that the T20 peptide has a tendency to insert itself in cellular membranes. Here we demonstrate that membrane-anchored T20 is able to inhibit Env-mediated cell-cell fusion, although low concentrations of the fusion inhibitor anchored to the membrane of target cells are not sufficient to prevent infection by free HIV-1 viruses. Given that HIV-1 has been reported to exit the cell through lipid rafts and incorporate raft-associated cellular proteins in its envelope during exit, we also analysed the infectivity of HIV-1 pseudotypes produced in T20-expressing cells. We found that ADA-T20 viruses are more infectious than viruses produced in control cells, which suggests that the propensity of the peptide to insert in the outer membrane leaflet of membranes may favour the anchoring of the virus to the target cell.

The second strategy involves the inhibition of dihydroceramide desaturase, the enzyme responsible for converting dihydroceramide to ceramide, with the ceramide analogue GT11. We show here that dihydroceramide desaturase inhibition results in the accumulation of saturated dihydrosphingomyelin in detergent-resistant membranes of GT11-treated cells. Although GT11 alters the lipid composition of l_o domains, it does not significantly affect the localization of raft-associated proteins, nor does it alter chemotaxis and antigen-induced activation of T cells. Nonetheless, we found that GT11 is able to prevent both Env-mediated cell-cell fusion and infection by free HIV-1 pseudotyped viruses in a dose-dependent manner. The replacement of sphingomyelin for dihydrosphingomyelin in lipid rafts of GT11-treated cells increases the rigidity of l_o domains, and these biophysical changes may explain the drug's antiviral activity. However, ADA viruses produced in GT11-treated cells are more infectious than those produced in untreated cells, which suggests that the antiretroviral effect of drugs that modulate membrane fluidity is complex and requires further investigation.

INTRODUCTION

1. The Human Immunodeficiency Virus Type I

1.1 AIDS - an emerging epidemic

The Acquired Immunodeficiency Syndrome, commonly referred to as AIDS, is a disease that affects more than 30 million people worldwide, according to the latest estimates from the World Health Organization (see www.unaids.org for recent data about the epidemic). The causative agent is the Human Immunodeficiency Virus (HIV), a retrovirus of the *Lentivirinae* subfamily first identified in 1983 by Luc Montagnier at the Institute Pasteur in France (Barre-Sinoussi et al., 1983; Hoffman, 2007; Levy et al., 1984). The virus primarily infects cells from the immune system that possess a CD4 receptor, such as T-lymphocytes and macrophages (Dalgleish et al., 1984; Landau et al., 1988; Maddon et al., 1986), and for that reason HIV induces a slow but steady decrease in the number of these cells in the organism. Indeed, individuals that are infected with HIV can live for several years without any discernible symptom, and only after the number of T-lymphocytes drops below a given threshold (normally below 200 cells per mm^3 of blood) does the person enter the so-called AIDS phase of the disease, with the appearance of opportunistic infections (tuberculosis, Kaposi's sarcoma, hepatitis, pneumonia) and a prognosis of death within 2-3 years (Hoffman, 2007; Kahn and Walker, 1998; Wei et al., 1995). Since the first cases of AIDS were reported in the early 1980's, the disease has been studied and better understood, with treatment plans developed over the years. Consequently, the life expectancy for an HIV-infected individual has increased greatly and AIDS has, to some degree, become a manageable disease. However, a cure has not been found yet and new strains of viruses resistant to the existing therapy are surfacing everyday.

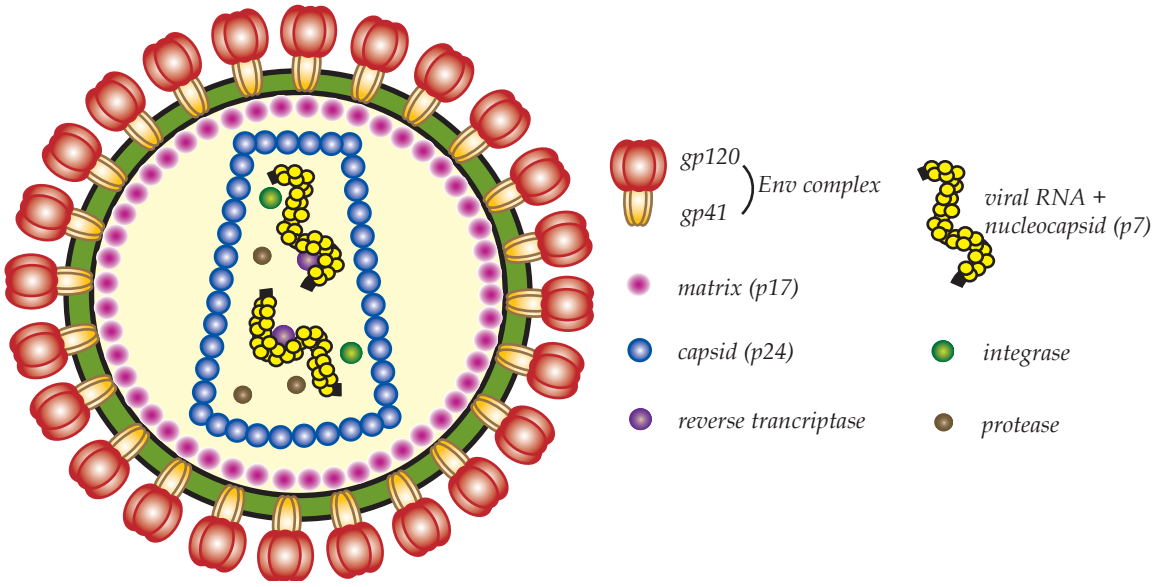


Figure 1. Structure of a HIV-1 viral particle.
Schematic representation of a mature HIV-1 virion.

1.2 Structure of the virus particle

HIV belongs to the *Lentivirinae* subfamily of retroviruses, having been identified so far two different species, HIV type 1 and HIV type 2. These show very different pathogenecity and, while HIV-2 is largely confined to West Africa, HIV-1 is highly disseminated globally and accounts for the vast majority of AIDS cases worldwide (Reeves and Doms, 2002). HIV-1 is characterized by a RNA genome in the form of two single-stranded molecules, which are composed by three main regions, *gag-pol-env*, flanked by long terminal repeat (LTR) regions necessary for the intermediate step in retrotranscribing the RNA genome into a DNA molecule (Sierra et al., 2005; Turner and Summers, 1999). Furthermore, the viral particle is around 100-120 nm in diameter and possesses a lipid envelope derived from the host cell membrane, surrounding a cone-shaped capsid composed of p24 proteins (Figure 1). Between the envelope and the capsid, subunits of the viral p17 protein comprise the matrix of the virion, confering it integrity, and inside the capsid we can find not only the nucleocapsid (p9 and p6 proteins closely attached to the RNA dimer) but also viral enzymes necessary for the replication cycle: the reverse transcriptase, the integrase and the protease. Embedded in the viral envelope are the so-called viral spikes (also called Env complexes), trimers of two non-covalently attached glycoproteins, gp120 and gp41. These play an important role in the entry of the virus in the cell (Sierra et al., 2005; Turner and Summers, 1999).

1.3 Replication cycle overview

The first step in the infection cycle of HIV-1 is the attachment of gp120 to the CD4 receptor at the surface of the target cell (Landau et al., 1988) (Figure 2). The virus is able to infect cells from the immune system that express the CD4 receptor, mainly T-lymphocytes and macrophages, although it has also been described to infect dendritic cells and CD4 negative cells, such as microglial cells of the central nervous system (Cosenza et al., 2002; Dalgleish et al., 1984; Engering et al., 2002; Maddon et al., 1986). Furthermore, in order to enter the cell HIV-1 also needs to attach itself to a second receptor, which was found to be a member of the seven transmembrane domain G protein-coupled receptors, or GPCRs (Deng et al., 1996; Feng et al., 1996). These receptors respond to chemokines, small peptides that function as potent chemoattractants for leukocyte subpopulations. HIV-1 hijacks either the CCR5 or the CXCR4 chemokine receptors for entering the target cell, although other chemokine receptors have also been described as coreceptors or as being required for entry (Deng et al., 1997; Rucker et al., 1997). According to the tropism of the virus for either coreceptor, HIV-1 has been classically categorized as follows: M-tropic strain, that uses the CCR5 receptor to infect macrophages, is mainly responsible for the initial stages in HIV-1 infection and is not associated with the formation of syncytia (fusion between Env-expressing cells and CD4+ cells, leading to the formation of giant multinucleated cells); T-tropic strain, that uses the CXCR4 receptor to

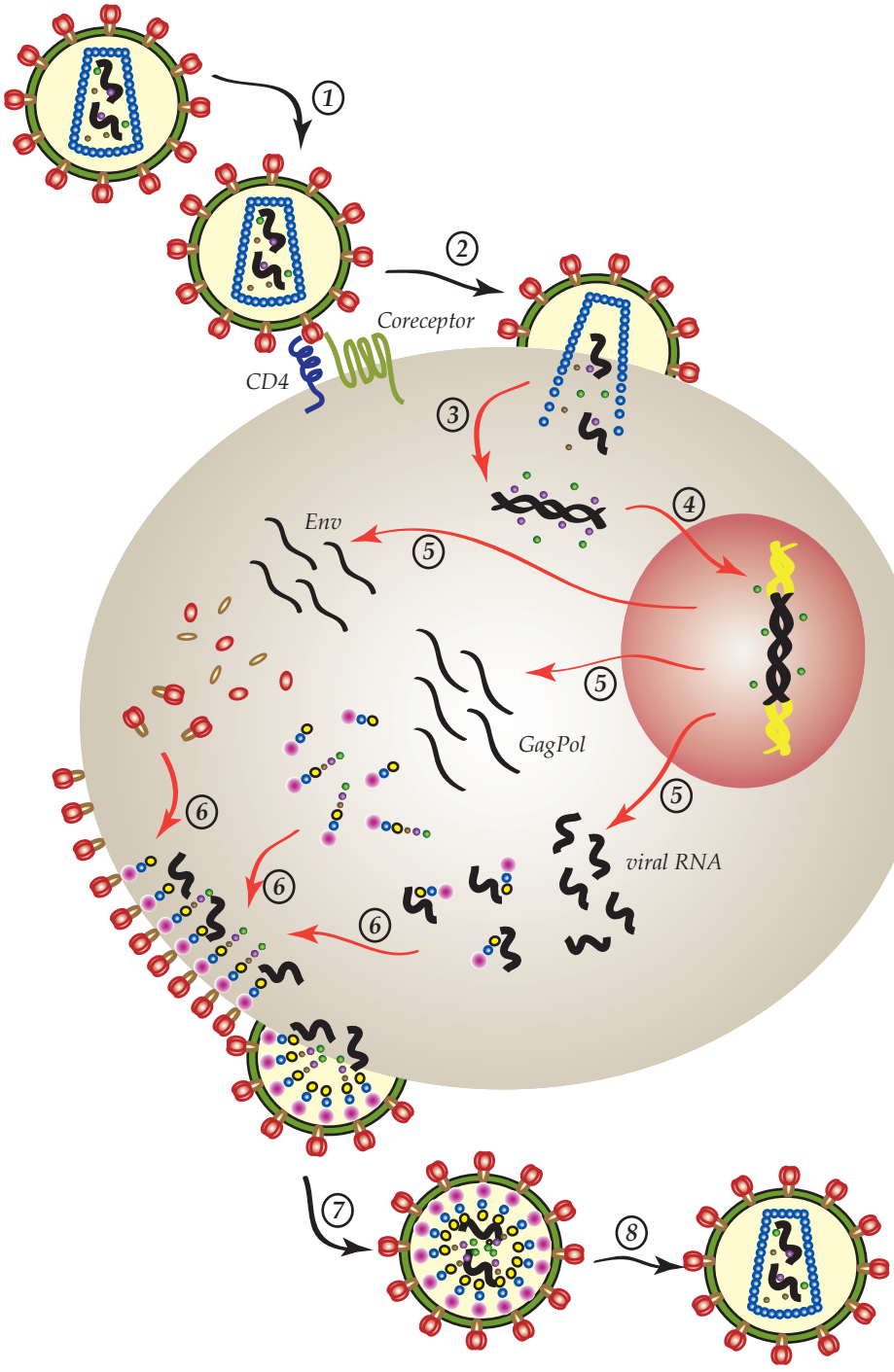


Figure 2. HIV-1 life cycle.
Schematic representation of the life cycle of HIV-1. (1) Attachment of the virus to cell receptors. (2) Fusion of the viral and cellular membranes, and release of the viral capsid inside the cell. (3) Retrotranscription of the single strand RNA into a double strand DNA. (4) Transport of the viral DNA into the nucleus and integration in the cell's genome. (5) Viral transcription and replication. (6) Assembly of viral particles. (7) Budding of viral particles from the cell. (8) Maturation of the virion.

infect T-lymphocytes, arises in the later stages of AIDS infection and is reported to induce the formation of syncytia; and last, dual-tropic strain, that can use either coreceptor to infect both macrophages and T-lymphocytes (Berger et al., 1998; Deng et al., 1996; Feng et al., 1996; Tersmette et al., 1988). After interacting with the chemokine coreceptor, the Env complex adopts a fusogenic conformation that permits the fusion of both the viral envelope and the membrane of the target cell, with the release of the viral capsid in the cytoplasm (Chan and Kim, 1998; Markosyan et al., 2003). This process will be discussed later.

The next step in HIV-1 replication cycle is the uncoating of the viral capsid with the subsequent retrotranscription of the RNA genome into a double-stranded DNA, a process mediated by the viral reverse transcriptase (Figure 2). At this stage, the viral protein Vif is essential to counteract the antiviral effects of the cellular protein APOBEC3G, a cytidine deaminase responsible for the introduction of C to U mutations in the negative strand of the DNA molecule. Vif downregulates APOBEC3G and prevents it from being integrated in nascent virions (Kozak et al., 2006; Sheehy et al., 2002). Once the viral genome is in the form of a DNA molecule, it enters the nucleus of the cell and the integrase mediates the insertion of the viral DNA into the cell's own genome. The virus can lie in a dormant state if the cell is not activated, since the first round of viral replication, that produces the regulatory proteins Tat, Rev and Nef, is mediated by cellular transcription factors such as NF-κβ (Hiscott et al., 2001; Nabel and Baltimore, 1987). When sufficient amounts of Tat are produced, it activates transcription of more Tat, Rev and Nef proteins through binding to the LTR region in the integrated viral DNA and other cellular transcription activators, inducing the production of multi-spliced mRNAs that remain retained in the nucleus of the cell (Feinberg et al., 1991; Ruben et al., 1989). Once the levels of Rev increase, this protein is able to bind unspliced or single-spliced mRNAs and transport them to the cytoplasm to the cell, where they will be translated in several viral proteins important for the assembly and release of new virions (Malim et al., 1989).

The mRNAs transported to the cytoplasm of the cell give rise to the precursor proteins gp160, Gag and Gag-Pol (Figure 2). Gp160, a result of the translation of the *env* gene, is glycosilated in the endoplasmic reticulum and proteolitically cleaved in two different glycoproteins, gp120 and gp41, in the Golgi apparatus. The glycoproteins anchor themselves in the membrane of the cell, in the final form of a homotrimer of heterodimers, with three subunits of each glycoprotein present in each Env complex (Earl et al., 1991). On the other hand, the Gag polyprotein is a 55 KDa protein that possesses the matrix, nucleocapsid and the capsid viral proteins (among others), and travels to the membrane of the cell where it becomes anchored (Burniston et al., 1999; Gottlinger et al., 1989; Sandefur et al., 2000). The Gag-Pol polyprotein, which results from a -1 frameshift in the transcription of the Gag precursor (Jacks et al., 1988), additionally contains the enzymes reverse transcriptase, integrase and protease, which will be incorporated in the newly-formed viral particles. The Gag and Gag-Pol precursors form multimers that travel and attach themselves to the inner leaflet of the cell membrane, also transporting genomic copies of HIV in the form of RNA molecules. The multimerization induces the outwards formation of roughly spherical structures that contain the RNA molecules and the Gag and Gag-Pol precursors inside, and eventually these structures give rise to immature virus particles that

leave the cell through a budding process, maintaining the cell membrane as its viral envelope with the Env complexes already anchored and several other cellular proteins (Gottlinger et al., 1989; Jouvenet et al., 2006; Nguyen and Hildreth, 2000). Once outside the cell, the virion undergoes maturation through the autoexcision of the protease from the Gag-Pol polyproteins and the subsequent processing of the remaining proteins in the viral precursors. The newly-formed viral particles are now fully infectious and ready to initiate a new replication cycle (Bukrinskaya, 2004; Ganser-Pornillos et al., 2008).

1.4 Current treatment options

Since the discovery of the causative agent of AIDS until the present day, many resources have been employed in the development of potentials drugs that are able to inhibit some step in the replication cycle of the virus, although a complete cure has not been achieved yet. Several classes of antiretroviral drugs are already available for treatment regimens, such as nucleoside/nucleotide or non-nucleoside inhibitors of the reverse transcriptase, inhibitors of the viral protease and inhibitors of the integrase (De Clercq, 2002). Since the virus has a high rate of mutation, due to the lack of proof-reading when retrotranscribing its genome, the emergence of resistant strains to the existing treatment is very high. While some of the acquired mutations are detrimental to the virus, others are beneficial in surpassing the obstacles created by the antiretroviral drugs (Martinez-Picado et al., 2000). This problem has been partially overcome by the combination of three or more inhibitors from at least two different classes of antiretroviral drugs, in what is called HAART, or Highly Active AntiRetroviral Therapy. This treatment plan has improved enormously the life expectancy of HIV-1-infected patients, by attacking the virus in more than one stage in its replication cycle, but there is a great need for further investigation of new targets of inhibition to combat newly acquired resistance by the virus (Hughes et al., 2008).

Recently, a new class of antiretroviral drugs became available in HAART treatment, usually used as a salvage therapy for patients that have developed multi-drug resistant strains of the virus. This new class targets the entry process (Starr-Spires and Collman, 2002), either by inhibiting the fusion of membranes mediated by gp41 (Enfuvirtide, which was approved for clinical use in 2003) (Matthews et al., 2004) or, in more recent times, by preventing the attachment of the virus to the CCR5 chemokine coreceptor (Maraviroc, which began clinical use in 2007) (Lieberman-Blum et al., 2008). Still under investigation are maturation inhibitors, drugs that prevent the processing of the Gag polyprotein and thus the production of mature capsid proteins, resulting in non-infectious viral particles (Salzwedel et al., 2007).

Another approach taken by the scientific community is the development of an HIV-1 vaccine, mostly as a preventive measure in populations in high risk of infection (Walker and Burton, 2008). However, this approach has proved fruitless over the past few years, as the main candidates AIDSVAX and V520 have shown no significant reduction in the incidence of HIV-1 infection in the participants of the clinical trials where the vaccines were administered

(indeed, some controversial data indicate that V520 may have even increased the risk of HIV-1 infection) (Francis et al., 1998; James, 2003; Priddy et al., 2008; Timberg, 2007). The reasons for the failure in the development of an effective HIV-1 vaccine are mainly due to the glycosilation of gp120, which masks the already highly mutable epitopes found in this glycoprotein (Huang et al., 2008).

2. Mechanism of HIV-1 entry into target cells

2.1 Attachment to the CD4 receptor

The entry of HIV-1 in T-cells is a complex, multi-step process that involves several cell factors and viral proteins. The first event to take place is the engagement by the virus of the CD4 receptor at the surface of the target cell (Dalgleish et al., 1984; Landau et al., 1988) (Figure 3). CD4 is a 55-60 KDa member of the immunoglobulin superfamily, that possesses four extracellular domains (D1, D2, D3 and D4), a hydrophobic transmembrane domain and a cytoplasmic tail (Maddon et al., 1985). The D1 domain is important to interact with major histocompatibility complex (MHC) class II molecules in antigen-presenting cells, and consequently CD4 acts as a costimulatory receptor in the formation of the T cell receptor (TCR) complex (Bowers et al., 1997; Rudolph et al., 2006). Furthermore, the intracellular cytoplasmic tail interacts with the tyrosine kinase Lck and induces a signalling cascade resulting in the activation of the cell (Bowers et al., 1997; Turner et al., 1990). In the case of HIV-1, the interaction between CD4 and the virus is mediated by the gp120 glycoprotein, present in each Env complex in the form of a trimer, non-covalently attached to three other gp41 subunits (Roux and Taylor, 2007). Gp120 possesses five conserved domains, buried inside the molecule, separated by five hypervariable regions; even though part of the molecule is exposed to the host's immune system, its high glycosilation and mutation rates allow the virus to escape neutralization by antibodies elicited against it (DeVico, 2007; Huang et al., 2008). After attaching itself to the D1 domain of the CD4 receptor, the gp120 subunits undergo conformational changes that allow them to interact with the second receptor necessary for cell entry, either CXCR4 or CCR5, depending on the viral strain (Kwong et al., 1998; Wu et al., 1996).

It should be mentioned that HIV-1, in certain instances, is able to infect cells that don't express the CD4 receptor, such as epithelial cells of the mucosal surfaces (through the glycosphingolipid galactosyl-ceramide, GalCer) (Yahi et al., 1992), cells from the nervous system (using the mannose receptor) (Liu et al., 2004), and dendritic cells (mostly through the C-type lectin DC-SIGN) (Geijtenbeek and van Kooyk, 2003; Turville et al., 2002). In the latter, the receptor DC-SIGN recognizes the high mannose content of gp120 and mediates the internalization, though not the infection, of HIV-1 in the cell. The dendritic cell then travels to the lymph nodes, matures, and transfers the still infectious virus to CD4+ T-cells, where it can initiate a productive infection (Engering et al., 2002; Wu and KewalRamani, 2006).

2.2 Attachment to a chemokine co-receptor

The second cell receptors to be engaged by the virus in the entry process are members of the seven transmembrane family of G protein-coupled receptors (Figure 3), responsible for the detection of chemotactic gradients and the ability of the cells to migrate towards them (Murdoch and Finn, 2000; Murphy, 1994). These receptors are characterized by a short N-terminal extracellular region, seven helical transmembrane domains with three extracellular and three intracellular loops, and a C-terminal cytoplasmic tail; the second intracellular loop has been reported to interact with G proteins upon receptor activation by the corresponding chemokine (Gilman, 1987; Moro et al., 1993). The chemokine receptors most used by the virus are CXCR4 (Feng et al., 1996), whose ligand is SDF-α (CXCL12), and CCR5 (Deng et al., 1996), which binds RANTES (CCL5), MIP-1α (CCL3), MIP-1β (CCL4) and CCL8. Indeed, these chemokines have been shown to be able to inhibit HIV-1 entry in the cell, by acting as viral competitors for the corresponding receptors (Bleul et al., 1996; Cocchi et al., 1995). However, there are reports of the virus using other GPCRs as co-receptors for entry, such as CCR3, CCR2b and CCR8 (Choe et al., 1996; Doranz et al., 1996; Rucker et al., 1997); nonetheless, these infections are rare and less efficient than the ones mediated by CCR5 and CXCR4.

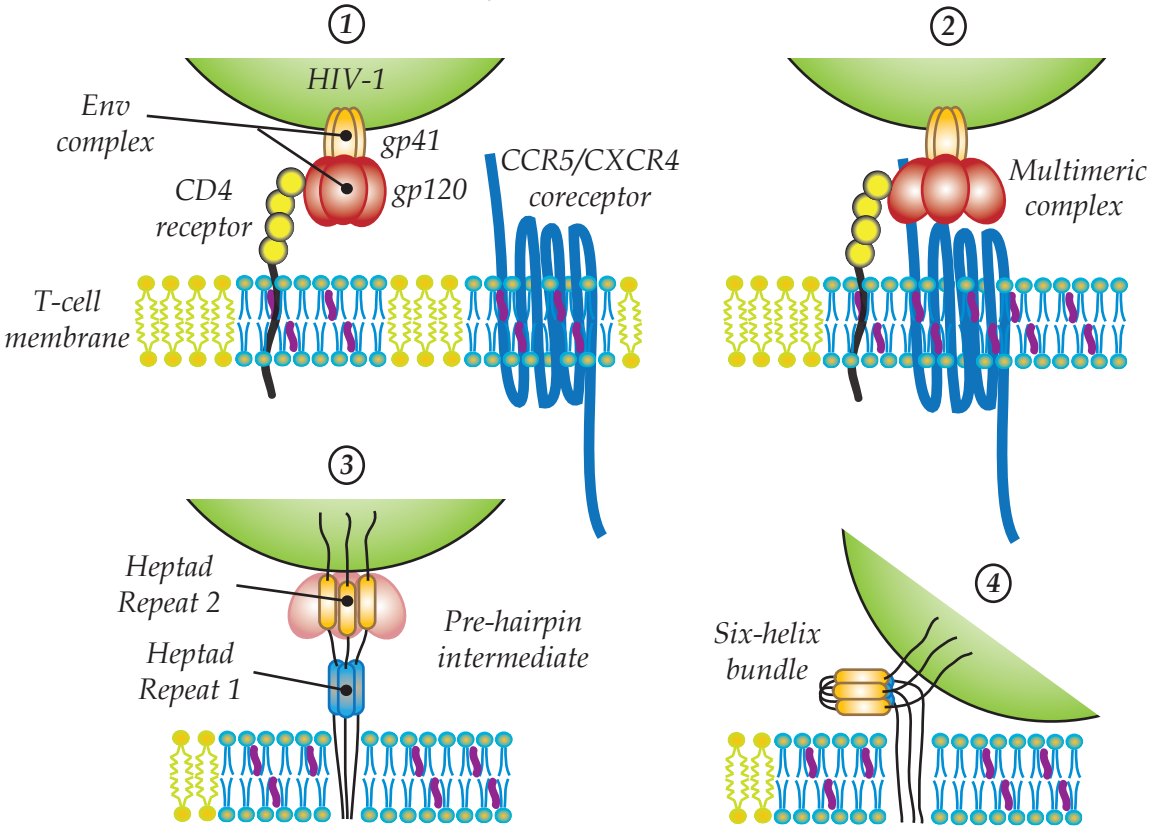


Figure 3. HIV-1 entry in the target cell.
Detailed scheme of the entry of an HIV-1 viral particle in the target cell. (1) Attachment of the gp120 glycoprotein to the CD4 receptor. (2) Gp120 conformational change and attachment to the chemokine coreceptor. Formation of a multimeric gp120-CD4-coreceptor complex. (3) Insertion of gp41 fusion peptides in the cell membrane. (4) Formation of a six-helix bundle and fusion of the viral and cellular membranes.

The conformation that gp120 adopts upon attachment to the CD4 receptor presumably exposes crucial regions necessary for the binding of the glycoprotein with either CXCR4 and/or CCR5 (Thali et al., 1993) (Figure 3). Studies have demonstrated the importance of the N-terminal region and the extracellular loops of the receptors in this interaction (Liu et al., 2003), and the relevance of the V3 variable region of gp120 in determining the tropism of the virus for either receptor (Cocchi et al., 1996; Hwang et al., 1991). This second receptor binding induces the dissociation of the gp120 trimer from the Env complex (Moore et al., 1990), and the consequent exposure of the gp41 subunits that leads to a third fusion-active conformation of the viral spike (Figure 3).

2.3 Fusion of the membranes

Following shedding of the gp120 subunits from the Env complex, the virus initiates the stage of membrane fusion mediated by the gp41 glycoproteins (Figure 3). Gp41 possesses an N-terminal ectodomain, a hydrophobic transmembrane region, and a C-terminal cytoplasmic tail; furthermore, the ectodomain consists of an aminoterminal nonpolar fusion peptide (FP) rich in glycines, two leucine zipper-like 4-3 regions consistent with the formation of α-helices (N-terminal heptad repeat 1, HR1, and C-terminal heptad repeat 2, HR2), and a hinge region between the heptad repeat regions that contains two important cysteine residues (Dong et al., 2001; Lu et al., 1995; Weissenhorn et al., 1997) (Figure 4). The next step in the entry process is the formation of a coiled-coil with the HR1 regions of the gp41 subunits, which thrusts the aminoterminal fusion peptides to insert themselves in the membrane of the target cell (Markosyan et al., 2003; Weissenhorn et al., 1997) (Figure 3). This causes the opening of a labile fusion pore in the cell membrane, one that can be closed if the fusion process is halted by low temperatures or inhibitory peptides (Markosyan et al., 2003). The gp41 molecules now adopt a fusogenic conformation and fold themselves, bringing together both the viral and the cell membranes and simultaneously allowing for the interaction between the HR1 and the HR2 regions. These contain complementary sequences that associate in an antiparallel manner in a structure called six-helix bundle (6HB), a cluster of six α-helices formed by the three HR1 and the three HR2 regions in each Env complex. The fusion pore becomes stable and the fusion of the membranes occurs, with the release of the viral capsid inside the cell (Chan et al., 1997; Markosyan et al., 2003) (Figure 3).

Figure 4. Structure of HIV-1 gp41 glycoprotein.
The gp41 glycoprotein is composed by a N-terminal fusion peptide, two heptad repeat regions separated by a hinge region with two cysteine residues, an hydrophobic transmembrane region, and a C-terminal cytoplasmic tail. FP, fusion peptide. TM, transmembrane region. CC, cysteine residues.

The entry process of the virus in the cell requires several factors in order to be successful. Studies have demonstrated that the density of the receptors for HIV-1 entry is essential for the establishment of a productive infection; multiple Env trimers are required to engage several CD4 and co-receptor molecules to activate the kinetics necessary for the fusion of the membranes (Kuhmann et al., 2000; Layne et al., 1990). Moreover, the conformation that the receptors adopt in the membrane and their affinity for gp120 are also relevant for HIV-1 binding and entry in the cell (Doms, 2000).

2.4 The fusion inhibitor enfuvirtide

At the stage where the fusion peptides of the gp41 subunits are already inserted in the membrane of the target cell, but the six-helix bundle has not yet formed, there is a pre-fusion conformation of gp41 (so-called pre-hairpin intermediate) which can last for several minutes before advancing into the full fusion-competent conformation (Markosyan et al., 2003). In this step, the HR1 regions are exposed and various C-peptides (derived from the HR2 region) have already been demonstrated to have the capacity to recognize the hydrophobic grooves on the surface of the coiled coil and inhibit the formation of the six-helix bundle, preventing altogether the fusion of the membranes (Chan and Kim, 1998; Wild et al., 1992; Wild et al., 1994) (Figure 5).

One such peptide, corresponding to the HR2 region of gp41 (residues 643-678 of HIV_{LAI} gp160), is enfuvirtide, previously named DP-178 and T20 (Kilby et al., 1998) (Figure 5). Early *in vitro* studies have demonstrated the efficacy of enfuvirtide in inhibiting HIV-1-induced syncitium formation with an IC_{90} of 1,5 ng/mL (Wild et al., 1994). Interestingly, the aminoacid residues complementary to enfuvirtide are only adjacent to the hydrophobic groove in HR1 to which other C-peptides strongly adhere (Chan et al., 1998); nonetheless, it shows more inhibitory efficacy than these (Kliger et al., 2001). Besides the accepted mode of action for these peptides (Kliger and Shai, 2000), some authors believe that enfuvirtide might also be able to bind the fusion peptide based on circular dichroism studies (Liu et al., 2005). Another interesting finding is the propensity that enfuvirtide shows to insert itself in the outer leaflet of liposomes, which might bear relevance to its inhibitory action *in vivo* (Kliger et al., 2001; Veiga et al., 2004); the membrane of the target cell, upon treatment with the drug, might act as an enfuvirtide reservoir and readily present the peptide when the virus approaches the cell.

Enfuvirtide has undergone several phase I/II/III clinical trials, in which its ability to significantly reduce viral loads in infected patients and partly restore CD4+ cell counts has been documented (Kilby et al., 1998; Lalezari et al., 2003a; Matthews et al., 2004). Moreover, two large-scale phase III clinical trials provided evidence that this compound is active against viral strains resistant to several other antiretroviral drugs (Lalezari et al., 2003b; Lazzarin et al., 2003; Matthews et al., 2004). Adverse effects associated with enfuvirtide treatment were reported, but for the most part were local injection site reactions and not considered severe enough to discontinue treatment (Lalezari et al., 2003b; Lazzarin et al., 2003). The results derived from

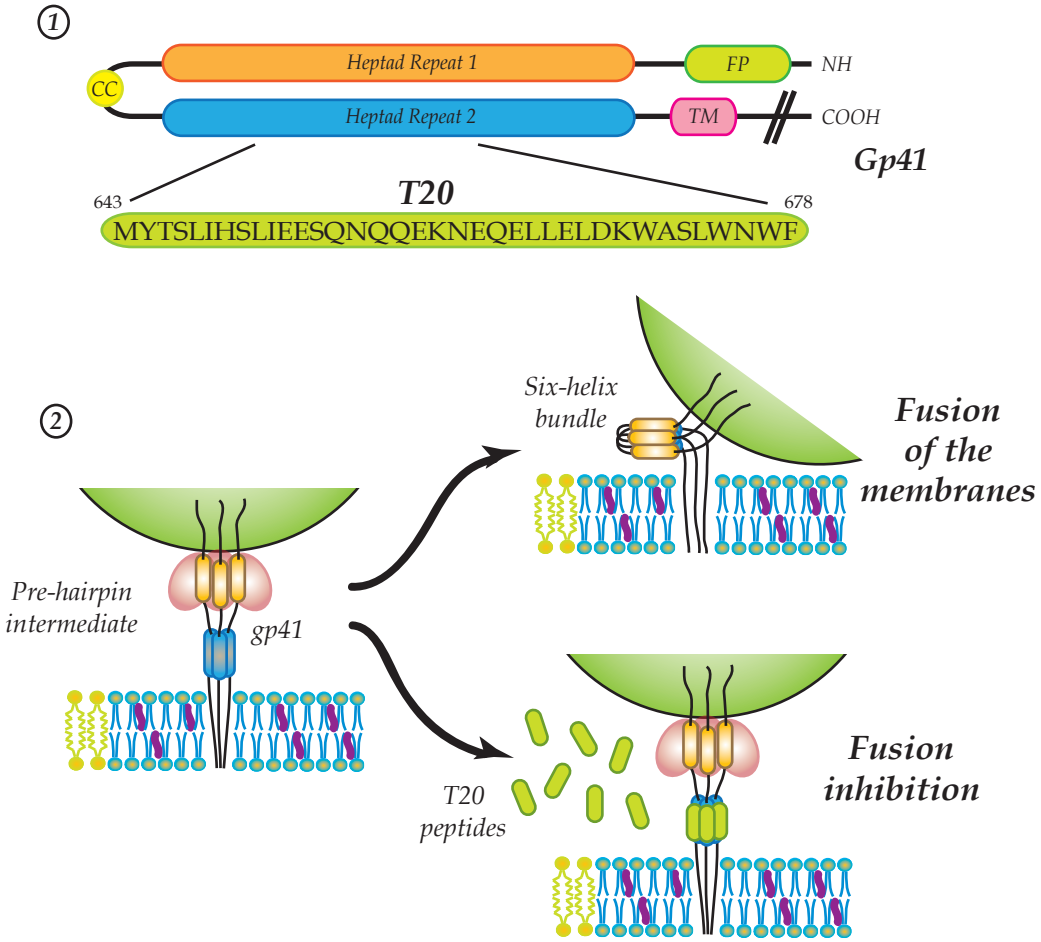


Figure 5. T20 mechanism of fusion inhibition.
(1) Gp41 aminoacid residues that correspond to the T20 peptide. (2) Mechanism of fusion inhibition by the T20 peptide. The T20 peptides recognize and attach to the complementary residues in the HR1 region and prevent the formation of the six-helix bundle.

the clinical trials have prompted the approval of the drug for commercial use in March 2003, and since then it has been successfully used as a salvage therapy for treatment-experienced patients.

The clinical trials and these recent years of enfuvirtide implementation on HAART have demonstrated that continued exposure to the drug leads to the emergence of resistant viral strains, similar to what happens with other classes of antiretrovirals (Matthews et al., 2004; Wei et al., 2002). Mapping studies indicated that the most common mutations that the virus undergoes to acquire resistance to enfuvirtide are aminoacid substitutions in residues 36-45, particularly in the GIV motif (residues 36-38) (Rimsky et al., 1998). In fact, due not only to the appearance of resistant strains but also to other issues associated with enfuvirtide treatment (lack of oral availability, high costs of manufacture, local injection site reactions) (Matthews et al., 2004), an Austrian-based group has demonstrated *in vitro* that a membrane-bound version of the peptide in target cells is able to inhibit HIV-1 entry, with the intention of using this strategy in future gene therapy approaches (Egelhofer et al., 2004).

3. Lipid rafts in the cell membrane

3.1 Structure and lipid composition

The fluid mosaic model proposed by S. J. Singer and Garth L. Nicolson to illustrate the arrangement of lipids and proteins in the membrane of the cell was first described in 1972, and remained the classical view of cell membrane structure until recently (Singer and Nicolson, 1972). In this model, the main components of the cell membrane are arranged in a random manner, with phospholipids forming a lipid bilayer and proteins, either integral or peripheral, distributed homogenously and capable of lateral diffusion without restrainment. Numerous studies have refined this view of cell membrane organization, mainly through the observation that non-ionic detergents, such as Triton X-100, are not able to completely solubilize the cell membrane at low temperatures. From these experiments came the notion of low-density detergent-resistant membranes (DRMs), or detergent-insoluble glycolipid-enriched complexes (DIGs), fractions of the cell membrane high in sphingolipids and cholesterol that partition from the rest of the phospholipid-rich membrane (Ahmed et al., 1997; London and Brown, 2000; Schroeder et al., 1998).

Studies have shown that the coexistence of (glycerol)phospholipids and sphingolipids in membranes induces the separation of these components into different phases, due to their structure (Figure 6). Phospholipids typically have kinked unsaturated acyl chains, with multiple double bonds and a low T_m, which results in a liquid-disordered (l_d) state of the membrane, while sphingolipids, with their saturated or mono-unsaturated acyl chains and high T_m, induce the formation of a highly ordered solid-like gel phase, completely segregated from the fluid state conferred by the phospholipids. However, cholesterol interacts preferentially with sphingolipids and alters the gel conformation of pure sphingolipid-domains to a liquid-ordered (l_o) phase, lowering the T_m and allowing for lateral mobility in the membrane (Bunge et al., 2008; Estep et al., 1979; Filippov et al., 2004; Frazier et al., 2007). These l_o domains found in the membrane of the cells, which account for the majority of isolated DRMs, are named lipid rafts and show an asymmetric nature, with sphingolipids, sphingomyelin and cholesterol enriched in the outer leaflet while cholesterol and phospholipids with saturated hydrocarbon chains (such as phosphatidylinositol and phosphatildylethanolamine) are predominant in the inner leaflet (Brown and London, 2000; Fridriksson et al., 1999; Simons and Ikonen, 1997).

A closer look at sphingolipid structure reveals that they consist of long chain bases (most commonly sphingosine) linked by an amide bond to a fatty acid, and to complex carbohydrate or polar head groups via their terminal hydroxyl group. Sphingomyelin and phosphatidylcholine, for instance, both possess phosphorylcholine as the polar head group (Ramstedt and Slotte, 2002), while gangliosides have instead a carbohydrate moiety containing sialic acids. The sphingosine unit modified by the long chain fatty acid is referred to as ceramide, a key intermediate in the synthesis of complex sphingolipids (Brown, 1998; Fantini et al., 2002). Ceramide is formed by the introduction of a *trans* 4,5 double-bond in

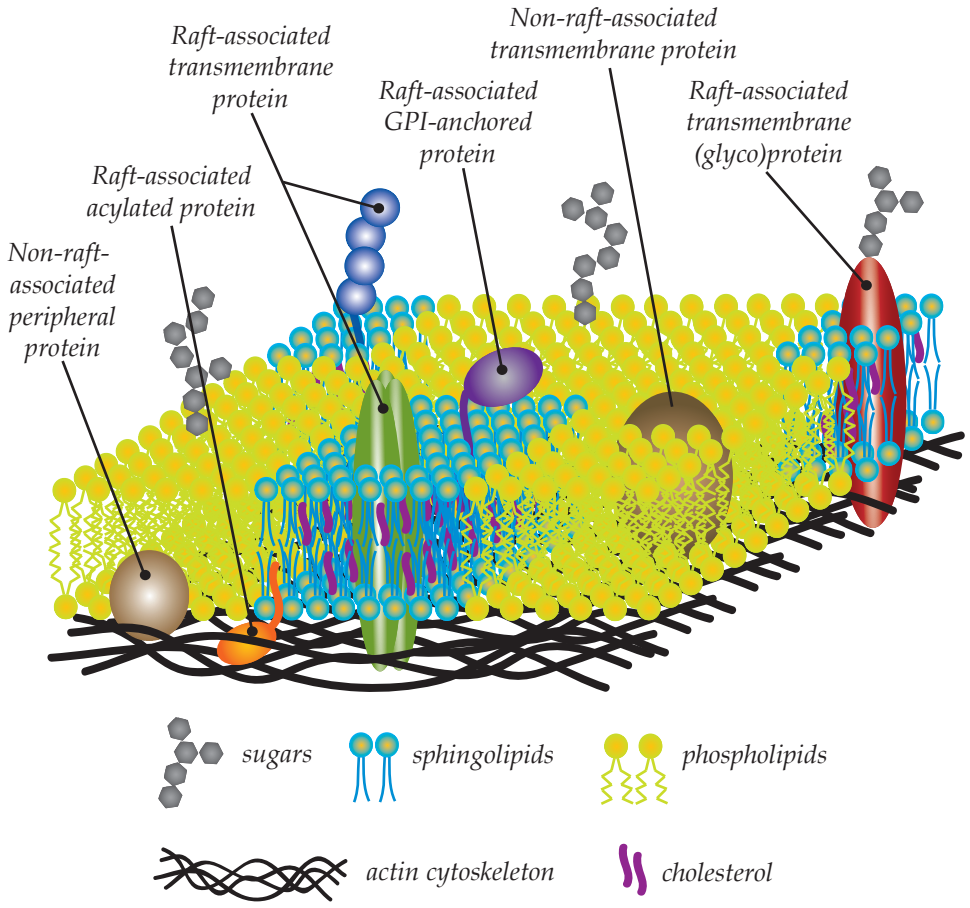


Figure 6. Lipid and protein composition of the cell membrane.

dihydroceramide, the direct saturated precursor of ceramide, by the enzyme dihydroceramide desaturase (DHCDase) (Figure 7). It will then give rise to various gangliosides in the membrane, sphingomyelin, and other sphingolipids of importance to the cell (Lahiri and Futerman, 2007; Rawat et al., 2005). Furthermore, the catabolism of sphingomyelin, mediated by the action of acid sphingomyelinase or phospholipase C, is also able to generate *de novo* ceramide; this effect is stimulated by numerous factors including apoptosis-inducing agents (irradiation, heat shock, toxins), which is in accordance with the well-documented implication of ceramide in the regulation of programmed cell death (Cifone et al., 1994; Santana et al., 1996; Zhang et al., 2001).

3.2 Protein composition

Several proteins are known to partition preferentially in lipid rafts, essentially taking advantage of its particular lipid composition. The glycosylphosphatidylinositol (GPI) anchor, that attaches peripheral proteins to the outer leaflet of the membrane, and both the myristate and palmitate acyl chains, that direct proteins to the inner leaflet of the membrane, are common raft-targeting signals used by the cell (Brown and Rose, 1992; Resh, 1999). Notable examples of

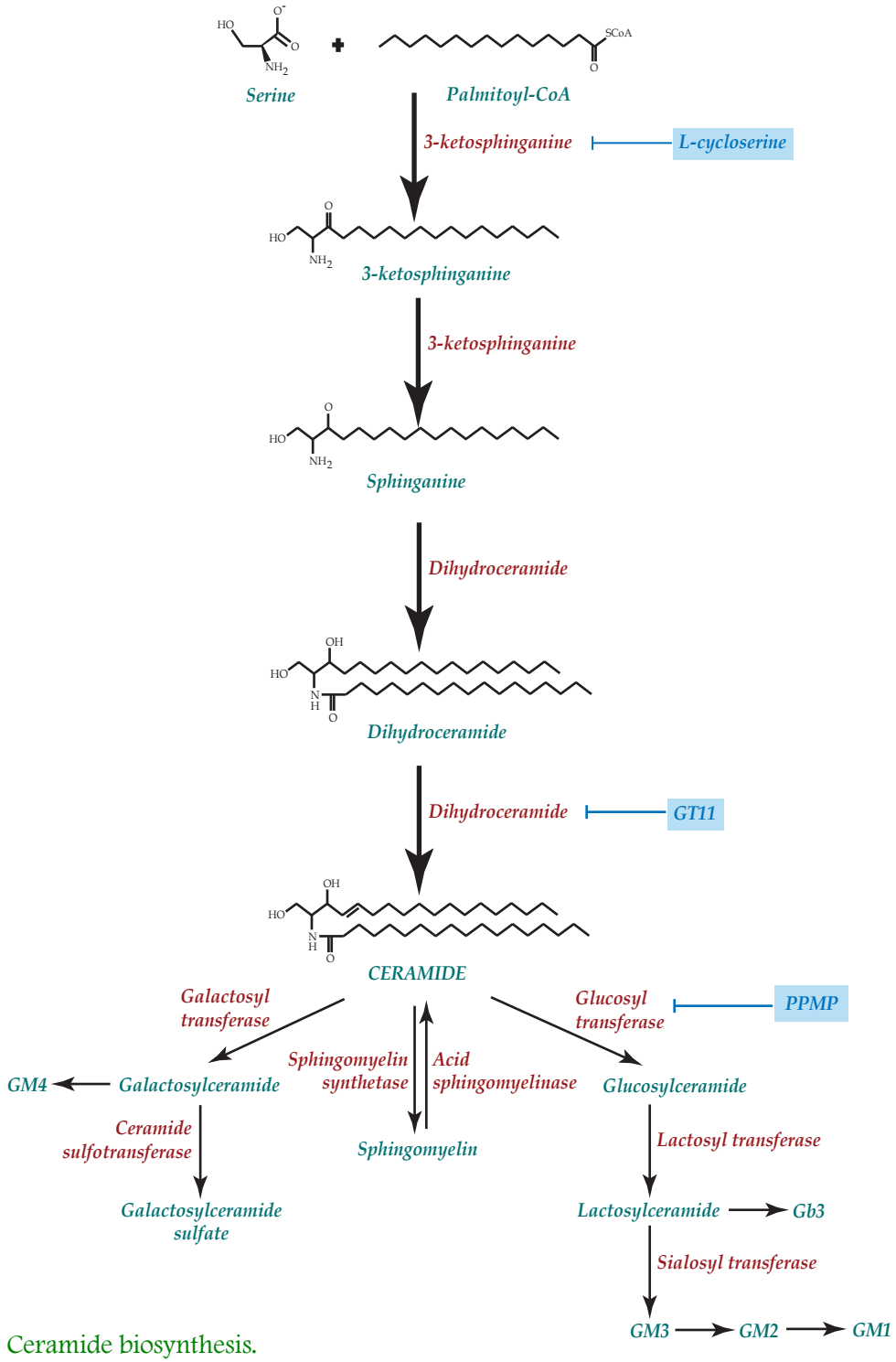


Figure 7. Ceramide biosynthesis.
Ceramide is formed from serine and palmitoyl-coA, and gives rise to sphingomyelin and gangliosides, among others.

proteins that use these signals to partition to lipid rafts are the CD14, CD24, CD55 and CD59 receptors with GPI anchors, and members of the Src family of tyrosine protein kinases, as well as the Gα subunits of heterotrimeric G proteins, that use the aforementioned palmitoylation signals (Moffett et al., 2000; Resh, 1994; Stefanova et al., 1991). On the other hand, prenylation seems to work as a raft-disfavouring signal, since its branched structure does not pack well in

the liquid-ordered arrangement of lipid rafts (Melkonian et al., 1999); indeed, it has been shown that, as mentioned above, the acylated Gα subunit of heterotrimeric G proteins associates better with lipid rafts than the full heterotrimeric Gαiβγ complex with its prenylated Gβγ subunits (Moffett et al., 2000).

Transmembrane proteins are less common in lipid rafts, mainly because the hydrophobic membrane-spanning domains do not fit well in its lipid structure (Lucero and Robbins, 2004). However, linkage of two or more palmitoyl chains to the cytoplasmic domain of transmembrane proteins has been shown to act as a raft-targeting signal in some proteins, such as the tetraspanin CD81 and the T-cell receptors CD4 and CD8 (Arcaro et al., 2000; Cherukuri et al., 2004; Fragoso et al., 2003). In fact, the function of the majority of lipid raft-associated transmembrane proteins is dependent on the palmitoylation signals they possess, implying a more direct role of these domains in the functionality of the cell other than a mere scaffold to which proteins attach.

3.3 Function in the context of the cell

Lipid rafts have the capacity to move laterally in the membrane, serving as dynamic platforms that coalesce with each other to form larger domains and facilitate interactions between previously separated proteins (Harder and Simons, 1997; Simons and Ikonen, 1997) (Figure 8). This property, commonly referred to as lipid raft clustering, is able to increase the stability and the initial size of individual rafts from 50-70 nm (or even less) to more than 500 nm for clustered domains (Gupta and DeFranco, 2003), as studies using single particle tracking and fluorescence resonance energy transfer (FRET) have shown (Kenworthy et al., 2000; Kusumi et al., 2004; Murase et al., 2004; Prior et al., 2003; Sharma et al., 2004). Several mechanisms have been implicated in lipid raft clustering, such as ligand binding to receptors, antibody crosslinking, or even cytoskeleton-induced interaction of proteins found in rafts (Harder et al., 1998; Rodgers and Zavzavadjian, 2001; Simons and Toomre, 2000). Furthermore, it has also been found that the aforementioned mechanisms have the ability to increase the raft affinity of a protein initially not associated with these domains, which in turn activates a cascade of events that enhances raft clustering (Field et al., 1995; Prieschl and Baumruker, 2000).

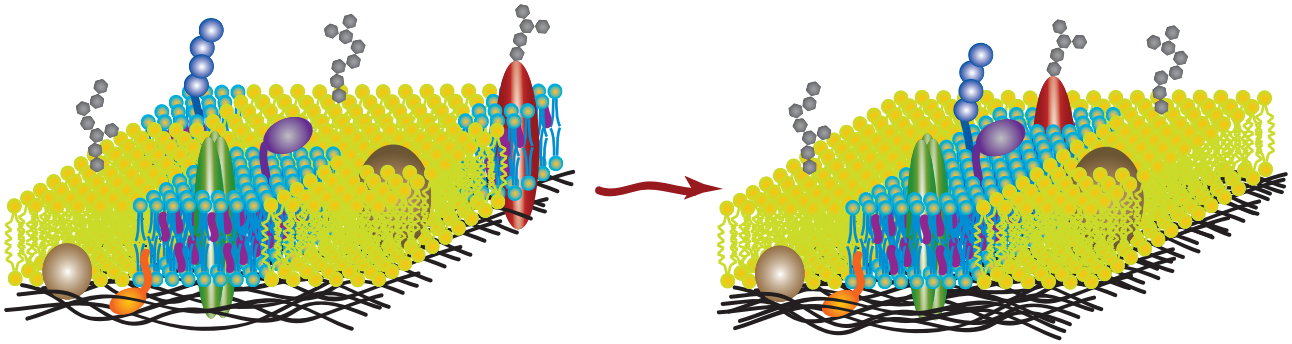


Figure 8. Clustering of lipid rafts at the cell membrane.

The transient or constitutive association of specific proteins with lipid rafts, and their capacity to coalesce in large platforms, are the basis of lipid raft implication in cell function (Brown and London, 2000; Simons and Ikonen, 1997). Rafts are important for such processes as cell proliferation, apoptosis, migration as well as infection by several pathogens, such as HIV-1 (Fantini et al., 2002; Grassme et al., 2001; Inokuchi et al., 2000; Manes et al., 2003; Manes et al., 1999). For instance, both p56Lck and LAT (Linker of Activated T cells) are crucial proteins involved in T-cell receptor-mediated signalling and their activity is dependent on their attachment to lipid rafts (Brdicka et al., 1998; Zhang et al., 1998a). Studies have shown that a mutant non-palmitoylated form of Lck that does not associate with lipid rafts is not able to interact with its signalling partners in the TCR complex and become phosphorylated, although the mutation does not render the protein inactive *per se*. Similarly, the transmembrane LAT protein cannot function in T-cell signalling if its palmitoylation site is absent and the protein is excluded from lipid rafts (Lin et al., 1999; Zhang et al., 1998b). Other examples describe how sphingolipid depletion in lipid rafts affects the localization of Src kinases and the overall proliferation capacity of mouse lung carcinoma cells (Inokuchi et al., 2000), and how raft disruption by sequestering cholesterol can impair the ability of a human breast cancer cells to migrate in response to a chemoattractant gradient (Liu et al., 2007).

Although the actin cytoskeleton is not directly associated with lipid rafts, its role in several cell functions depends on rafts, such as lymphocyte migration and activation (Manes and Viola, 2006; Viola and Gupta, 2007). By rearranging actin filaments, the cell is able to induce lateral movement of raft-associated transmembrane proteins anchored to them and create supramolecular clusters necessary for a given cell function. For instance, in TCR signalling, ligand binding induces the formation of an immunological synapse at the contact region between the T-cell and the antigen-presenting cell (APC); this immunological synapse is composed of a variety of T-cell receptors and signalling molecules (Lck, ZAP70, LAT, SLP76), which undergo a complex relocation due to the reorganization of the actin cytoskeleton (Chichili and Rodgers, 2007; Meiri, 2004; Valensin et al., 2002).

Another object of interest for these microdomains is their implication in disease; several pathogens, from viruses to bacterial toxins, are reported to use lipid rafts as sites of entry and even exit from the target cell (Chazal and Gerlier, 2003; Simons and Ehehalt, 2002; van der Goot and Harder, 2001). More specifically, it has been postulated that lipid rafts provide high amounts of low-affinity receptors to which the pathogens attach, further stabilize them and in some cases induce a conformational change in the pathogen's structure, necessary for a subsequent interaction with a high-affinity receptor less abundant in the membrane (Montecucco, 1986). Notable examples are the binding of cholera toxin to the raft-based ganglioside GM1 (Badizadegan et al., 2000), the interaction of Shiga toxin to the Gb3 glycolipid found in lipid rafts (Lingwood, 1999), and the attachment of tetanus and botulinum toxins to several di- and tri-sialogangliosides present in lipid rafts of neural cells (Kitamura et al., 1999; Simpson and Rapport, 1971; van Heyningen, 1974). In the case of the Shiga toxin, not only do lipid rafts act as binding sites to the target cell, but they also transport the toxin inside the cell into the endoplasmic reticulum, through clathrin-dependent endocytosis (Falguieres et al., 2001).

Additionally, lipid rafts have also been implicated in the budding of influenza virus from the target cell (also in HIV-1, as will be discussed ahead), in efficient prion conversion that takes place in Creutzfeldt-Jakob disease, and in the formation of amyloid plaques in Alzheimer disease (Fantini et al., 2002; Kakio et al., 2001; Nayak et al., 2004; Sanghera and Pinheiro, 2002; Zhang et al., 2000).

3.4 Role of lipid rafts in HIV-1 infection

It has been suggested that HIV-1 takes advantage of the existence of lipid rafts, and their importance in the cell, in various stages during its replication cycle. Not only the entry and exit of the virus from the cell take place in lipid rafts, but also viral regulatory proteins specifically interact with these domains with the intention of affecting cell signalling and promoting the production of infective virions (Campbell et al., 2001).

Shortly after infecting a new host, HIV-1 needs to penetrate a barrier of mucosal epithelial cells in the gastrointestinal, anorectal or genitourinary tracts. Since these cells do not express the receptors necessary for HIV-1 entry in the cell, the virus has taken advantage of a common mechanism of macromolecule transport in epithelial cells, transcytosis, to pass from the apical to the basolateral side of these cells without actually infecting them (Bomsel, 1997). Lipid rafts are important in this respect because the virus binds to raft-based GalCer to gain entry into the cell, and disrupting this association prevents viral transcytosis (Alfsen et al., 2001; Yahi et al., 1992). Also the productive infection of immune cells, through binding of the virus to the CD4 receptor and a chemokine coreceptor (either CXCR4 or CCR5), is a process in which lipid rafts play an essential role. Studies have shown that these receptors are localized in raft microdomains, and that the clustering ability of lipid rafts is crucial for the entry of the virus in the cell (Manes et al., 2000; Nguyen et al., 2005; Popik et al., 2002). Targeting CD4 to non-raft portions of the membrane does not impair the attachment of gp120 to this receptor, but the trimeric gp120-CD4-coreceptor complex, necessary for posterior fusion events, does not form (Del Real et al., 2002). The reason lies in the fact that binding of gp120 to raft-based CD4 triggers lateral diffusion and coalescence of individual rafts, bringing together gp120-CD4 complexes with rafts that possess either CXCR4 or CCR5, and if CD4 is localized outside rafts clustering does not occur (Del Real et al., 2002) (although opposite results have also been reported (Percherancier et al., 2003; Popik and Alce, 2004)). Furthermore, some studies refer that the gp120 glycoprotein also shows binding affinity to sphingolipids found in lipid rafts, particularly the ganglioside GM3 and the globotriaosylceramide Gb3, which might assist in HIV-1 entry into the cell (Hug et al., 2000; Puri et al., 2004); this data helps consolidates the notion that lipid rafts are fundamental for a productive infection to take place.

Once inside the cell, the virus continues to use lipid rafts to its benefit. Several viral proteins are reported to bind to these domains and alter cell signalling to accommodate vital changes necessary for the production of infective viral particles. One notable example is Nef, a peripheral, myristoylated membrane protein that interacts with raft-associated

nonreceptor tyrosine kinases of the Src family. It induces T-cell activation in the absence of stimulation, by clustering via lipid rafts such signalling molecules as Lck, Fyn and Hck, and subsequently promotes viral replication (Wang et al., 2000). It has also been described its effect in downregulating CD4 expression in the cell, allowing for efficient incorporation of Env in the viral particle (Lama et al., 1999) and avoiding superinfection of infected cells (Wildum et al., 2006). Furthermore, Nef inhibits apoptosis of the infected cell, by reducing intracellular p53 concentration and activation (Greenway et al., 2002), and decreases the expression of MHC class I molecules on the cell surface, effectively rendering the infected cell undetectable to CD8+ cytotoxic T-cells (Schwartz et al., 1996).

Other viral proteins that possess either myristoylation and/or palmitoylation signals that target them to lipid rafts are the Env glycoprotein complex and the Gag and Gag-Pol precursors. Env is synthesized as a 160 KDa precursor (as was mentioned before), that posteriorly undergoes cleavage and palmitoylation in the Golgi apparatus (Earl et al., 1991; Rousso et al., 2000). There is evidence that indicates that lipid rafts are also assembled in Golgi and subsequently travel to the cell membrane where they become incorporated (Smart et al., 1999); through the palmitoylation signals, it is possible that the Env complex interacts with these lipid raft precursor structures still in the Golgi apparatus and later is incorporated in the membrane of the infected cell, where the budding of new virions will occur (Nguyen and Hildreth, 2000). Also the Gag and Gag-Pol precursors are myristoylated in the p17 matrix subunits, which directs them to the internal leaflet of lipid rafts (Ono and Freed, 1999), and the oligomerization that these precursors undergo in the assembly of new virions helps cluster more raft units with gp120-gp41 complexes embedded (Lindwasser and Resh, 2001).

Finally, as would be expected, the budding of viral particles from the cell takes place in lipid rafts (Nguyen and Hildreth, 2000). Analyses of HIV-1 envelope lipid composition of infectious virions have shown a high content in cholesterol and sphingolipids, as opposed to phospholipids (Aloia et al., 1993). Additionally, treatment of viruses with β-cyclodextrin, a cholesterol-chelating agent, results in non-infectious viral particles, demonstrating the importance of lipid raft structure and composition to the virus (Campbell et al., 2002). In accordance with these data, a number of cellular proteins associated with lipid rafts have been detected in viral particles, such as CD55, CD59 and MHC class II molecules; the virus benefits from this fortuitous coincidence by interfering with virus-specific humoral responses and possibly stabilizing its attachment with the target cell, through CD4-MHC class II interactions (Arthur et al., 1992; Cantin et al., 1997; Esser et al., 2001; Lamarre et al., 1989; Montefiori et al., 1994).

3.5 Artefact or real?

Since the concept of lipid rafts was first introduced in the scientific community, researchers have been divided in the question of whether these microdomains really exist in the cell membrane or are merely an artefactual result derived from detergent extraction

at low temperatures (Edidin, 2001; Munro, 2003). In fact, the definition of detergent-resistant membranes comes from a biochemical operation, and given that phase-separation is strongly dependent on temperature (Bunge et al., 2008), new methods and techniques to isolate and/or visualise lipid rafts were in need to prove their existence in physiological conditions.

Still on the subject of membrane solubility with a non-ionic detergent, one major problem with Triton X-100 is the need for chilling before extraction, which presumably alters lipid distribution in the membrane and can lead to artificial raft formation and clustering (Heerklotz, 2002). The discovery of Brij 98 as an alternative detergent that can be used at 37ºC, has brought new insight into this technique. Rafts isolated with Brij 98 still possess much of the same proteins detected in Triton X-100 rafts, and interestingly, their size was around 70 nm, which probably corresponds to individual rafts (Drevot et al., 2002). Yet another detergent, Lubrol WX, is also used in cold extraction, although the lipid domains isolated with this detergent possess a different composition than the ones isolated with Triton X-100 (Delaunay et al., 2008; Lucero and Robbins, 2004; Roper et al., 2000); far from disconcerting, this evidence points towards the existence of distinct types of raft units involved in separated cell functions, that behave differently when subjected to extraction with different detergents (Schuck et al., 2003).

The visualization of rafts in cells has always been an appealing yet complicated challenge to overcome. The small size of these microdomains makes it difficult to detect them by microscopy, and therefore the most usual method applied is the clustering of several raft units by antibodies against known raft-associated markers (proteins or lipids) (Harder et al., 1998). A common example, although in this case not involving a protein localized in lipid rafts, is the usage of the B subunit of cholera toxin (CTx) as a binding agent and cross-linker of the ganglioside GM1 found in lipid rafts; this technique is extensively used in fluorescence microscopy (some examples in (Manes et al., 2000; Popik et al., 2002)). Additionally, FRET and single-particle tracking experiments have provided evidence that a GPI-anchored protein is organized in clusters and transiently confined to domains sensitive to a glycosphingolipid synthesis inhibitor, consistent with lipid rafts (Kenworthy and Edidin, 1998; Kenworthy et al., 2000; Sharma et al., 2004; Sheets et al., 1997). Furthermore, another group has constructed proteins with different transmembrane domains, to direct them either to raft or non-raft portions of the membrane, and using single-molecule tracking has concluded that the diffusion of raft-associated proteins is significantly reduced when compared to their non-raft protein counterparts, and additionally that cholesterol depletion minimizes those differences (Pralle et al., 2000). Although some researchers argue that sequestering cholesterol is a drastic move that may affect not only the composition of the membrane but also the correct functioning of the cell (Edidin, 2001; Pike and Miller, 1998), the use of glycosphingolipid synthesis inhibitors provides the same indication that these experiments interfere with lipid rafts in the membrane, as was mentioned above (Sheets et al., 1997). Overall, and given the assigned important roles that lipid rafts have in the context of the cell, growing evidence is surfacing everyday to support the existence of these microdomains in the membrane.

OBJECTIVES

1. Target T20 chimeric peptides to the external leaflet of cell membranes and analyse their effect in HIV-1 infection.

1.1 Generate stable cell lines that express raft-associated or raft-excluded membrane-anchored T20.

1.2 Analyse the effect of membrane-bound T20 in Env-mediated cell-cell fusion.

1.3 Analyse the effect of membrane-bound T20 in infection by free HIV-1 pseudotypes.

1.4 Determine the infectivity of HIV-1 pseudotypes produced in T20-expressing cells.

2. Inhibit the enzyme dihydroceramide desaturase (DHCDase) and analyse its effect in HIV-infection.

2.1 Determine the cellular toxicity of the DHCDase inhibitor GT11, and its derivative GT11pyr.

2.2 Analyse the effect of GT11 in Env-mediated cell-cell fusion.

2.3 Analyse the effect of GT11 in infection by free HIV-1 pseudotypes.

2.4 Study the effect of GT11 in T cell-associated functions.

2.5 Analyse the lipid composition of lipid rafts in GT11-treated cells.

2.6 Study the localization of raft-associated proteins in GT11-treated cells.

2.7 Study the biophysical properties of dihydrosphingomyelin-containing membranes.

2.8 Determine the infectivity of HIV-1 pseudotypes produced in GT11-treated cells.

MATERIALS AND METHODS

General procedures

Cell lines used and cultivation methods

The adherent cell lines HEK-293T, HEK-293, HEK-293CD4 (that contains a stably transfected plasmid that encodes for the CD4 receptor) and TZM-b1 (a HeLa-derived cell line that expresses high levels of CD4, CCR5 and CXCR4) were cultured in DMEM medium (Lonza BioWhittaker) with 10% FCS, 2mM L-glutamine, sodium pyruvate and antibiotics (complete medium), unless explained otherwise. HEK-293CD4 cells were also cultured in the presence of G418 (1 mg/mL), to select for cells that express the CD4 receptor. The Jurkat suspension cell line was maintained in RPMI medium (Lonza BioWhittaker) with 10% FCS, 2 mM glutamine, sodium pyruvate, antibiotics and non-essential aminoacids (complete medium), unless mentioned otherwise .

Peripheral blood mononuclear cells (PBMCs) were obtained from healthy donors through centrifugation at 2000 *g* for 30 min in a Ficoll-Paque gradient (Pharmacia). After centrifugation a thin layer of PBMCs (buffy coat) is recovered and washed twice to remove platelet contamination. The PBMCs were then stimulated *in vitro* with phytohemaglutinin-L (PHA-L; 1 μg/mL; Difco) for 24h at 37ºC, in complete RPMI medium plus human interleukin 2 (IL-2; 20 ng/mL; Hoffman-LaRoche). PHA-L was removed from the medium and the cells were maintained in culture with the remaining components, unless detailed otherwise.

Effect of membrane-bound T20 in HIV-1 infection

T20LDL and T20GPI cloning procedures

To generate a construct that encodes a membrane-bound version of T20 localized outside lipid rafts, the extracellular (a short sequence), transmembrane and juxtamembrane low-density lipoprotein receptor (LDLR) sequences, the cytoplasmic region of CD46, a small signal peptide that directs the chimera to the membrane and the T20 peptide were cloned together. The T20 sequence present in a previously available T20GPI-containing vector (gift from Patrick Keller) was first amplified using the primers P/T20-KPNI/FW and P/T20-HINDIII/RV, and cloned in frame in the pLGFP-GT46 vector (P. Keller, Max Planck Institute of Cell biology and Genetics, Dresden, Germany), yielding T20-LDL-CD46. After several intermediate steps, this construct was then introduced in a bicistronic pRV-IRES-GFP vector (Genetrix, Madrid, Spain), along with a short signal peptide sequence, to yield SP-T20-LDL-CD46 (further referred to as T20LDL). The primer sequences are shown in Table I.

To generate a construct with the T20 peptide, the sequence encoding for a

glycosylphosphatidylinositol (GPI) anchor and a short signal peptide, the SP-T20-GPI sequence from the aforementioned T20GPI-containing vector was cloned in pRV-IRES GFP, to yield SP-T20-GPI (T20GPI in future references).

Table I. Primers used to clone T20LDL.

P/T20-XMNI/FW	5' TATGGTACCCATGTACACAAGCTT 3'
P/T20-HINDIII/RV	5' TACAAGCTTGAACCAATTCCACAG 3'

Generation of T20-expressing stable cell lines

HEK-293T cells were transfected by either calcium phosphate method or JetPEI (Poly Transfection) with pVSVG, a vector that codifies for the VSVG envelope (Genetrix, Madrid, Spain), pGag-Pol, that possesses the rest of the viral proteins necessary for the formation of retroviral particles (Genetrix, Madrid, Spain), and either one of the retroviral constructs pRV-IRES-GFP+T20LDL or pRV-IRES-GFP+T20GPI. 48h after transfection the viral supernatants were recollected, filtered, concentrated (ultracentrifugation at 50000 *g* for 2h, 4ºC, in a Beckman Coulter centrifuge), and infected in either HEK-293T cells or HEK-293CD4 cells. The resulting cell lines were sorted in a Coulter Epics Altra cytometer (Beckman Coulter) for GFP expression and, whenever necessary, for T20 expression using the 2F5 antibody (2 ug/mL; provided by Dr. Hermann Katinger through the NIH AIDS Research References Reagents Program) and a secondary anti-human IgG-PE antibody (Beckman Coulter).

Immunostaining for T20 presence in the membrane

T20LDL and T20GPI stable cell lines were stained for expression of T20 in the membrane with the 2F5 antibody (2 ug/mL) for 1h at 4ºC, followed by incubation (also 1h, 4ºC) with a secondary anti-human IgG-PE antibody (Beckman Coulter). The cells were further analised by flow cytometry.

For measuring the propensity of T20 to insert itself in the lipid bilayer of cells, HEK-293CD4 cells were incubated with either 0, 0.1, 0.5, 1, 2 or 6 μg of T20 peptide (provided by the Proteomics Service of the Centro Nacional de Biotecnologia, Madrid, Spain) per 2×10^5 cells for 24h at 37ºC. The cells were then stained with 2F5 antibody for 30 min at 4ºC and analised by flow cytometry.

Detection of T20 in lipid rafts using DRM isolation

T20LDL- and T20GPI-expressing HEK-293CD4 stable cell lines were lysed at 4ºC for 20 min in TNEX buffer (50 mM Tris-HCL pH 7.4, 150 mM NaCl, 5 mM EDTA, 0.5% Triton X-100) plus protease and phosphatase inhibitors (aproteinin, leupeptin, PMSF, $NaVO_4$, NaF). The cell lysates were mixed with an Optiprep solution (Nycomed Pharma, Oslo, Norway) at 60% to yield a 35% final concentration. After adding a 30% Optiprep solution to the 35% lysate-Optiprep mix in SW-41 centrifuge tubes (Beckman Instruments, Inc., Palo Alto, CA), detergent-resistant membranes (DRMs) were isolated by ultracentrifugation (170000 *g*, Beckman Coulter) for 4h at 4ºC. Six fractions were recovered from the ultracentrifugation, the first two ones corresponding to DRMs, and the last ones containing the remaining cellular proteins. Normalized protein amounts determined for each fraction were resolved in a SDS-PAGE gel and transferred to nitrocellulose membrane (Pharmacia) for western-blot analysis. Blotting was performed with the 2F5 antibody (2 ug/mL), for T20 detection, anti-transferrin receptor (0,25 μg/mL; Zymed Laboratories) and anti-caveolin-1 (0,4 μg/mL; Santa Cruz Biotechnologies, Inc.), as negative and positive controls for raft-associated proteins, respectively.

Immunofluorescence (copatching) experiments for visualization of T20 in the membrane

T20LDL and T20GPI stable cell lines (HEK-293T) were placed in fibronectin-coated chambers (10 ug/mL), allowed to adhere for 4h at 37ºC, and incubated with the 2F5 antibody (2 ug/mL) for 30 min at 12ºC. After washing twice with DMEM + 0.2% BSA (at 4ºC), the cells were incubated with anti-human IgG-Cy5 antibody (Jackson ImmunoResearch, Inc.) for 30 min at 12ºC, and halfway into the incubation cholera toxin subunit β-biotin antibody (6 μg/mL; Sigma Aldrich), against GM1, was added to the cells. Another washing step preceded the final incubation with streptavidin-Cy3 antibody (Jackson ImmunoResearch, Inc.), 30 min at 12ºC. The cells were fixed with 3,7% paraformaldehyde (5 min at 4ºC) and cold methanol (5 min at 4ºC), mounted in Vectashield medium (Vector Laboratories) and analysed by confocal microscopy (Olympus). The images were processed using Image J software.

Env-mediated cell-cell fusion assays using T20-expressing cell lines

T20LDL- and T20GPI-expressing HEK-293CD4 stable cell lines, HEK-293 and HEK-293CD4 cells were transfected (using JetPEI) with a pSCluc plasmid containing the firefly luciferase gene under the control of the vaccinia virus 7.5 promoter (provided by D. Rodriguez, Centro Nacional de Biotecnología, Madrid, Spain), and pNull promoterless renilla luciferase plasmid (gift from Gustavo del Real). A vaccinia virus that codifies for HIV-1env_{IIIB} was infected

in HEK-293 effector cells for 1h at 37ºC, and the cells were further maintained in culture for 12h in the presence of 100 μg/mL rifampicin (Boehringer Mannhem). 36h after transfection, the infected HEK-293 cells were co-cultured with the transfected cells (in 1:2 ratio) in the presence of rifampicin, for 6h at 37ºC. To the transfected HEK-293CD4 cells was also added either 0, 0.5, 1, 2 or 6 μg/mL of T20 peptide 24h before co-culturing with the infected cells, and the peptide was maintained throughout the 6h-long incubation. Moreover, the CXCR4-antagonist AMD3100 (10 μM; Sigma Aldrich) was added to the transfected HEK-293CD4 cells for 5 min at 37ºC previous to the co-culture with the infected cells, as a positive control for fusion inhibition. Cell-cell fusion was analysed by luciferase activity measurement in cell lysates (Passive Lysis Buffer, from Promega), by use of a Dual Luciferase Reporter Assay System (Promega). Relative Light Units (RLUs) were calculated as the quotient between firefly and renilla activity values, and were indicative of the occurrence of fusion between the transfected and the infected cells.

Production of replication-deficient HIV-1 pseudotypes and infection in T20-expressing cells

To generate replication-deficient HIV-1 viruses pseudotyped with different envelopes, HEK-293T cells were transfected (JetPEI or Calcium Phosphate Transfection kit, Invitrogen) with the pNL4.3*luc*R-E- vector (gift from Rafael Delgado, Hospital 12 de Octubre, Madrid, Spain), an HIV-1 expression vector modified with the luciferase gene (inserted in the *nef* gene) and deficient in *env* and *vpr*, and an expression vector that codifies for a given viral envelope: pADA*env* (an R5 HIV-1 envelope), pNL4.3*env* (an X4 HIV-1 envelope) or pVSVG*env* (from the vesicular stomatitis virus). All vectors were provided by Rafael Delgado. 48h after transfection the viral supernatants were recollected, centrifuged to remove cell contamination, and stored in aliquots at -80ºC. An ELISA for p24 detection (Innogenetics) was performed in order to quantify the viral supernatants.

To perform an infection assay, TZM-b1 cells were transfected with either pRV + T20LDL or pRV + T20GPI for expression of T20 at the membrane, and that expression was monitored by flow cytometry using the 2F5 antibody (2 μg/mL). The cells were then plated in p24 wells for infection, and 36h after transfection with the T20-expressing vectors, viral supernatants corresponding to 30 ng of p24 antigen, in the case of ADA and NL4.3, or 8 ng in the case of VSVG, were added to the cells for 24h at 37ºC. The medium was replaced and 48h later the cells were lysed (Passive Lysis Buffer) and infection was assessed by luciferase activity measurement. TZM-b1 cells were also incubated 24h before infection with 0, 2, 5 or 10 μg/mL of T20 peptide, which was renewed when the infection was initiated.

Production of replication-deficient HIV-1 pseudotypes in T20-expressing cells and infection in TZM-B1 cells

To generate replication-deficient HIV-1 viruses that express T20 in their envelopes, T20LDL and T20GPI stable cell lines (HEK-293T) were transfected with the pNL4.3*luc*R-E- vector and either pADA*env*, pADA*env* 38E/42S (with a two-aminoacid substitution that confers resistance to T20-mediated inhibition), pADA*env* 38A/42T (another two-aminoacid substitution that confers resistance to T20-mediated inhibition), pNL4.3*env*, pNL4.3*env* 38E/42S (the same two-aminoacid substitution as in pADA*env* 38E/42S) and pVSVG*env*. All vectors were a kind gift from Rafael Delgado. In addition, HEK-293T were also transfected to produce control viral supernatants for posterior infection assays. 48h after transfection the viral supernatants were recollected, centrifuged to remove cell contamination and stored in aliquots at -80ºC. An ELISA for p24 detection (Innogenetics) was performed in order to quantify the viral supernatants.

To perform an infection with the viral supernatants derived from T20-expressing cells, TZM-b1 cells were plated in p24 wells 24h before infection. The cells were also incubated with 0, 2, 5 or 10 μg/mL of T20 peptide, which was renewed when the infection was initiated. Viral supernatants corresponding to 25-30 ng of p24 antigen, or 8 ng in the case of pVSVG*env*, were added to the cells for 24h at 37ºC. The medium was replaced and 48h later the cells were lysed (Passive Lysis Buffer) and infection was assessed by luciferase activity measurement.

Effect of the inhibition of dihydroceramide desaturase (DHCDase) in HIV-1 infection

Viability and cell cycle analyses

Activated human PBMCs were incubated with 0, 0.1, 0.5, 1 or 2 μM of GT11 C8 (gift from Gemma Fabriàs, IIQAB, Barcelona, Spain) for 3 consecutive days, with GT11 C8 being replaced once daily. The cells were, on the one hand, counted every day using a Trypan Blue dye exclusion method to stain for dead cells, and on the other hand fixed with 70% ethanol at -20ºC for cell cycle analysis. The fixed cells were then stained with DNA Prep (Beckman Coulter) and analysed by flow cytometry.

Jurkat cells and activated human PBMCs were incubated for 3 consecutive days with 0, 1, 2, 5, 10, and 15 μM of GT11pyr (gift from Gemma Fabriàs), in the case of Jurkat cells, or 0, 0.5, 1, 2, 5 and 10 μM of GT11pyr, in the case of PBMCs (GT11pyr was replaced twice daily). As with GT11 C8, all the cells were counted every day and stained for cell cycle analysis. Additionally, HEK-293 cells were also incubated with 0, 15, 30 or 60 μM of GT11pyr, and the

cells were counted every day using Trypan Blue.

Citotoxicity assays

TZM-b1 cells were plated in p96 wells and incubated for 24h with 0, 1, 2, 4, 8, 16, 32 or 64 μM of GT11pyr. Cell toxicity was assessed by adding MTS (Promega) to the p96 wells for 2-4h before measuring absorbance at 492 nm. LD_{50} was determined as the concentration that corresponds to half the absorbance level of control wells.

Migration assays using modified Boyden chambers

In vitro migration assays were performed using modified Boyden chambers (Costar, Cambridge, MA), in which a chemotactic gradient is formed between two separate compartments communicated by a porous membrane. Chambers with a pore size of 5 μm were used for Jurkat cells, and with a pore size of 3 μm for activated human PBMCs.

In the case of Jurkat cells, cells were incubated with either 0.2 or 2 μM of GT11 C8 in basal medium for 24h. The cells were then placed in the open chamber of each well while the lower part received either complete medium, basal medium, or basal medium + 10 nM human SDF-1α (Prepotech, Inc.). GT11 C8 was renewed in both the upper chambers and in the wells. After 3-4h at 37ºC, the cells that had passed through the pore membrane into the lower part of the well were recovered and counted by flow cytometry. Additionally, Jurkat cells were also incubated with 0, 1, 2, 5 or 10 μM of GT11pyr for 8h in complete medium and overnight in basal medium (RPMI + 0,1% BSA), with renewal of GT11pyr once. The rest of the procedure continued as described above.

In the case of PBMCs, cells were incubated with 0, 0.5, 1, 2 or 5 μM of GT11pyr in complete medium + IL-2 for 24h before depleting the cells in basal medium for 3h, renewing GT11pyr once during the 24h-incubation and again in the depletion period. The rest of the procedure continued as described above.

Analysis of PBMCs activation through quantification of IFNγ production

Non-stimulated human PBMCs (right after Ficoll-Paque separation, before stimulating with PHA-L and IL-2) were incubated with 0, 0.5, 1, 2, 5, or 10 μM of GT11pyr, in complete RPMI medium without FCS, for 36h at 37ºC. GT11pyr was replaced twice during the incubation period. The cells were then plated in p96 wells coated with anti-CD3 antibody (5 μg/mL; eBioscience) and anti-CD28 antibody (5 μg/mL; Pharmingen), in order to activate them, and incubated for 12h at 37ºC (in the presence of GT11pyr). The supernatant of the wells was

recollected and quantified for IFNγ using an human IFNγ ELISA kit from R&D Systems.

DRM isolation for determination of lipid composition and CD4 localization

HEK-293CD4 cells were incubated for 24h at 37ºC with 0, 15, 30 or 60 μM of GT11pyr, which was renewed once during the incubation period. Afterwards, cells were lysed and DRM fraccionation was performed as detailed above (see "***Detection of T20 in lipid rafts using DRM isolation***"). Of the six fractions recovered from the ultracentrifugation, half was processed for lipid composition analysis, using High Performance Liquid Chromatography (HPLC) coupled to time of fly (TOF) mass spectrometry (HPLC-MS), and the remaining half was analysed for CD4 localization by western-blot. Blotting was performed with anti-CD4 antibody (1 μg/mL; Santa Cruz Biotechnology, Inc.), anti-transferrin receptor (0,25 μg/mL; Zymed Laboratories) and anti-caveolin-1 (0,4 μg/mL; Santa Cruz Biotechnology, Inc.), the last ones serving as negative and positive controls for raft-associated proteins, respectively.

Immunofluorescence (copatching) experiments for visualization of raft-associated proteins

HEK-293CD4 cells were transfected (JetPEI) with a vector encoding for a GFPGPI construct (P. Keller, Max Planck Institute of Cell biology and Genetics, Dresden, Germany), and the following day the cells were incubated with either 0 or 60 μM of GT11pyr, for an additional 24h (GT11pyr was renewed once). The cells were then placed in fibronectin-coated chambers and allowed to adhere for 4h at 37ºC, in the presence of GT11pyr. Afterwards, the untransfected HEK-293CD4 cells were incubated for 30 min at 12ºC with either anti-CD4 antibody (on one occasion HP2.6, 20 μg/mL, and on another occasion anti-CD4 coupled to FITC, [], from Immunotech) or anti-CXCR4 antibody (K1046; Santa Cruz Biotechnology, Inc.), and 10 minutes before the end of the incubation to all cells (including to the GFP-GPI transfected ones) was added cholera toxin subunit β-biotin antibody (6 μg/mL; Sigma Aldrich). The cells were then washed twice with DMEM + 0,2% BSA (at 4ºC), and incubated for an additional 20 min at 12ºC with anti-mouse IgG-Cy2 antibody (Jackson ImmunoResearch, Inc.) in the case of the CD4 wells, anti-rabbit IgG-Alexa Fluor 488 antibody (Molecular Probes) in the case of the CXCR4 wells, and streptavidin-Cy3 antibody (Jackson ImmunoResearch, Inc.) for all wells. The cells were fixed with 3,7% paraformaldehyde (5 min at 4ºC) and cold methanol (5 min at 4ºC), mounted in Vectashield medium with DAPI (Vector Laboratories) and analysed by confocal microscopy (Olympus). The images were processed using Image J software. (Olympus).

Env-mediated cell-cell fusion assays using GT11-treated cells

HEK-293 and HEK-293CD4 cells were transfected (using JetPEI) with a pSCluc plasmid containing the firefly luciferase gene under the control of the vaccinia virus 7.5 promoter, and pNull promoterless renilla luciferase plasmid. 24h after transfection, the transfected HEK-293CD4 cells were also incubated with 0, 0.01, 0.1, 0.5, 1, 5 or 10 μM of GT11 C8 for an additional 24h. A vaccinia virus that codifies for HIV-1env_{IIIB} was infected in HEK-293 effector cells for 1h at 37ºC, and the cells were further maintained in culture for 12h in the presence of 100 μg/mL rifampicin (Boehringer Mannhem). Afterwards, the infected HEK-293 cells were co-cultured with the transfected cells (in 1:2 ratio) in the presence of rifampicin, for 6h at 37ºC. Cell-cell fusion was analysed by luciferase activity measurement in cell lysates (Passive Lysis Buffer, from Promega), by use of a Dual Luciferase Reporter Assay System (Promega). Relative Light Units (RLUs) were calculated as the quotient between firefly and renilla activity values, and were indicative of the occurrence of fusion between the transfected and the infected cells.

Production of replication-deficient HIV-1 pseudotypes and infection in GT11pyr-incubated cells

To generate replication-deficient HIV-1 viruses pseudotyped with different envelopes, HEK-293T cells were transfected with the pNL4.3*luc*R-E- vector and either pADA*env*, pNL4.3*env* or pVSVG*env*. 48h after transfection the viral supernatants were recollected, centrifuged to remove cell contamination, and stored in aliquots at -80ºC. An ELISA for p24 detection (Innogenetics) was performed in order to quantify the viral supernatants.

To perform an infection in cells incubated with high doses of GT11pyr, TZM-b1 cells were incubated with either 0, 15, 20 or 30 μM of GT11pyr for 24h, during which GT11pyr was renewed once. Viral supernatants corresponding to 45 ng of p24 antigen were added to the cells for 24h at 37ºC (GT11pyr incubation was maintained but not renewed). The medium was replaced and 48h/72h later the cells were lysed (Passive Lysis Buffer). Infection was assessed by luciferase activity measurement.

To perform a "chronic treatment" of TZM-b1 cells with low concentrations of GT11pyr, the cells were incubated with either 0, 0.5, 1, 1.5 or 2 μM of GT11pyr in complete medium with 5% FCS for 36h, 60h or 84h before infection (GT11pyr was replaced twice daily). Viral supernatants corresponding to 25 ng of p24 antigen were added to the cells for 24h at 37ºC (GT11pyr incubation was maintained but not renewed). The medium was replaced and 48h later the cells were lysed (Passive Lysis Buffer). Infection was assessed by luciferase activity measurement.

Production of replication-deficient HIV-1 pseudotypes in GT11pyr-incubated cells and infection of TZM-B1 cells

To generate replication-deficient HIV-1 viruses pseudotyped with different envelopes, HEK-293T were transfected with the pNL4.3*luc*R-E- vector and pADA*env*. 12h after transfection the cells were incubated with either 0 or 30 μM of GT11pyr, which was maintained for a further 36h (GT11pyr was renewed once). Afterwards, the viral supernatants were recollected, centrifuged to remove cell contamination, concentrated (ultracentrifugation at 52000 *g* for 2h, 15ºC, in a Beckman Coulter centrifuge) and stored in aliquots at -80ºC. An ELISA for p24 detection (Innogenetics) was performed in order to quantify the viral supernatants.

To perform an infection with the viral supernatants derived from GT11pyr-incubated cells, TZM-b1 cells were plated in p24 wells 24h before infection. Viral supernatants corresponding to 35 ng of p24 antigen were added to the cells for 24h at 37ºC. The medium was replaced and 48h later the cells were lysed (Passive Lysis Buffer). Infection was assessed by luciferase activity measurement.

RESULTS

1. Effect of membrane-bound T20 in HIV-1 infection

The discovery of the T20 peptide as a novel inhibitor in HIV-1 infection was a scientific breakthrough in HIV-1 research. This peptide has been shown to inhibit HIV-1-induced syncitium formation at low concentrations (IC_{90} of 1,5 ng/mL, (Wild et al., 1994)), presumably by interacting with the HR1 region in the gp41 glycoprotein complementary to its aminoacid residues and effectively preventing the formation of the six-helix bundle (Chan and Kim, 1998; Wild et al., 1992; Wild et al., 1994).

1.1 Analysis of the insertion of gp41-derived T20 peptide in the membrane of target cells

Previous studies have suggested the propensity of T20 to insert itself in the outer leaflet of liposomes, which might be important to its inhibitory action *in vivo*. Indeed, it has been suggested that the membrane of the target cell might act as a reservoir of T20 molecules that are present at the site of entry of HIV-1 in the cell, in an unspecific manner (Veiga et al., 2004). To gain further insight on the ability of soluble T20 to insert in the membrane of target cells, a synthetic version of the T20 peptide was added to HEK-293CD4 cells for 24h. After washing, cells were stained with the 2F5 antibody, a specific antibody that recognizes a gp41 epitope present in T20 (provided by Dr. Hermann Katinger through the NIH AIDS Research and References Reagents Program). Flow cytometry analysis confirmed that T20 was bound in the membrane of the cells, as is shown in Figure 9. Furthermore, a dose-dependent increase of the soluble peptide was observed; cell incubation with increasing concentrations of the T20 peptide shifted fluorescent curves to the right. These results suggest that the T20 peptide inserts in the membrane of target cells.

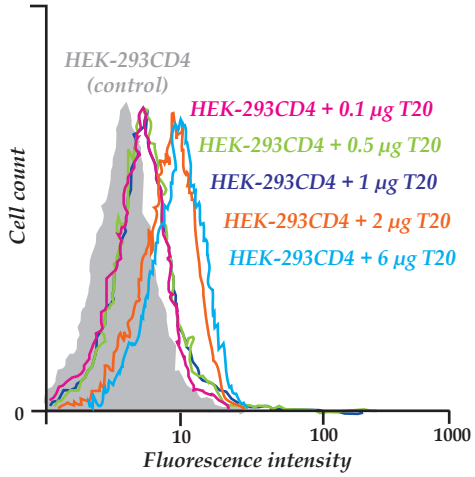


Figure 9. Soluble T20 peptide is able to insert itself in the membrane of cells.
HEK-293CD4 cells were incubated with 0, 0.1, 0.5 or 1 µg of T20 peptide for 24h at 37ºC. Staining for T20 in the membrane was performed using 2F5 mAb, and the cells were analysed by flow cytometry.

1.2 Generation and expression of membrane-anchored versions of T20 peptide

The results described above prompted us to direct T20 to the membrane of HIV-1 target cells, specifically inside or outside lipid rafts. In 2001, Hildinger (Hildinger et al., 2001) published the expression of T20 in the membrane of target cells as a mechanism to inhibit infection. By anchoring T20 in lipid rafts, instead of expressing it unspecifically in the membrane, we are allegedly increasing the concentration of the inhibitor in the site of viral entry, since it has been well described that HIV-1 uses raft microdomains as entry platforms (Manes et al., 2000; Nguyen et al., 2005; Popik et al., 2002), and additionally we are providing further evidence for the importance of lipid rafts in HIV-1 infection. The lipid raft-anchored construct will be compared with a similar construct localized outside raft microdomains, and both will be tested in fusion and infection assays to assess for their inhibitory capacity.

In order to express the T20 peptide at the membrane of the cell, two different T20 constructs were generated. On one hand, T20LDL was generated by cloning the sequence that codifies for the T20 peptide in frame with a short signal peptide, a short sequence of the ectodomain, the transmembrane and the juxtamembrane region of the low-density lipoprotein receptor (LDLR; this receptor is localized outside lipid rafts), and the cytoplasmic region of the CD46 receptor; this construct thus possesses the signals to be anchored in the membrane, specifically in a region outside lipid rafts. On the other hand, a T20GPI construct was also used, but in this case T20 was already provided in frame with a short signal peptide and the sequence that codifies for a glycosylphosphatidylinositol (GPI) anchor (gift from Patrick Keller), yielding T20GPI; as was mentioned before, the GPI anchor has the ability to attach proteins to the outer leaflet of lipid rafts, and thus T20GPI was used with the intent to be localized in these microdomains in the membrane. Both the LDL and the GPI targeting domains were previously used to target the CD4 receptor to non-raft and raft membranes, respectively (Del Real et al., 2002). The aminoacid sequence of the T20 constructs is described in Figure 10.

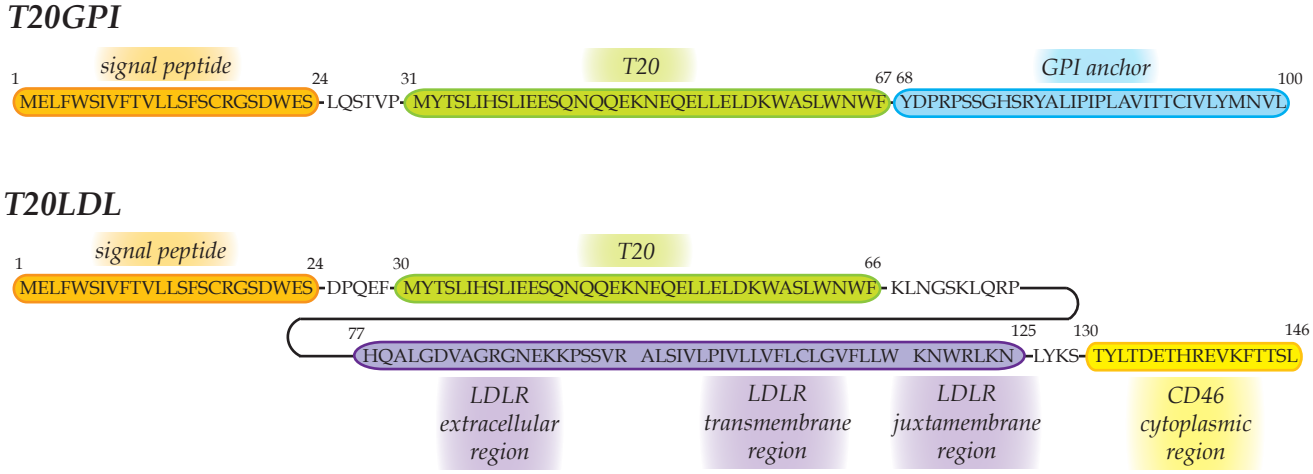


Figure 10. Aminoacid sequence of T20LDL and T20GPI constructs.

To generate cells that express membrane-bound T20LDL and T20GPI in a stable manner, T20LDL and T20GPI were both cloned in the bicistronic retroviral vector pRV-IRES-GFP and then transfected in HEK-293T cells (along with a vector that codifies for a VSVG envelope), in order to produce retroviral particles that were further used to infect cell lines. This infection induced the insertion of the retroviral genome in the cell's own DNA and the stable expression of the constructs at the membrane of the infected cell. Two different cell lines were used for the infection, HEK-293CD4 and HEK-293T, due to specific characteristics that each cell line possesses that best suited the following experiments. After infection the cells were stained with the 2F5 mAb. To enrich the cell population in T20-expressing cells, sorting was performed when less than 70% of cells were positive for T20 expression. Overall, the stable cell lines generated had between 70% and almost 100% cells that expressed T20 at the membrane, as shown in Figures 11A and 11B.

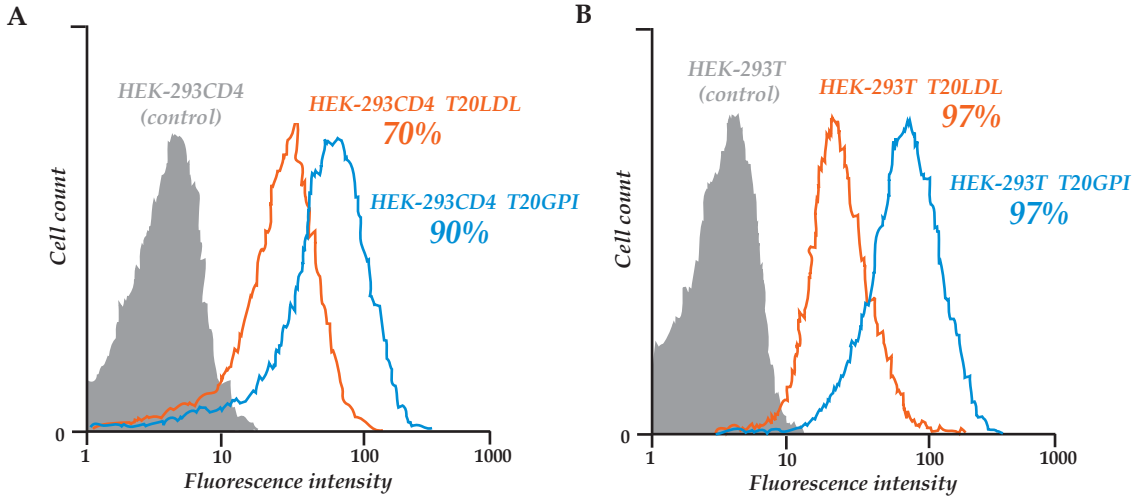


Figure 11. T20LDL and T20GPI are expressed at high levels in the membrane of stable cell lines.
T20LDL and T20GPI stable cell lines were stained for T20 expression in the membrane using 2F5 mAb. Flow cytometry analysis for stable cell lines produced in HEK-293CD4 cells (A) and in HEK-293T cells (B). In both (A) and (B) the percentages relative to positive staining are indicated.

1.3 Analysis of the localization of T20 constructs

We used two main approaches to check if each construct was being directed to the desired site in the membrane: isolation of detergent-resistant membranes (DRMs) and immunofluorescence (copatching) experiments.

- ***DRM isolation***

To perform this experiment, T20LDL- and T20GPI-expressing stable cell lines (HEK-293CD4) were lysed with Triton X-100 at 4ºC and ultracentrifuged in an Optiprep gradient. From the ultracentrifugation 6 different fractions were recovered which contained membrane fractions separated by their density. Membranes in the liquid-ordered state are resistant to solubilization by non-ionic detergents, such as Triton X-100, and "float" in the density

gradient formed upon centrifugation; as a result, the first two fractions recovered correspond to detergent-resistant membranes (DRMs) and are supposedly enriched in raft-associated proteins (these two fractions were pooled before assayed by western-blotting); the final fraction corresponds to solubilized proteins. In order to detect the localization of each T20 construct, the aforementioned 2F5 antibody was used in western-blot assays. The human receptor for transferrin was used as a negative control for proteins associated with lipid rafts, located at the bottom of the gradient (5th lane; Figure 12), whereas caveolin was used as a positive control for proteins associated with raft microdomains, located in the first two fractions (1st lane). Unfortunately, the 2F5 antibody recognized an unspecific band at the molecular weight where the T20 constructs were expected (around 12 KDa for T20LDL and 8 KDa for T20GPI), but it is clear from Figure 12 that a band from T20GPI cell lysates, presumably T20GPI, can be spotted in the same lane as the majority of caveolin, which indicates that at least a fraction of T20GPI partitions to raft microdomains in the membrane. However, given the unspecificities recognized by the 2F5 mAb, no specific band corresponding to the T20LDL protein was distinguished (Figure 12); nonetheless,we did not detect any band in the DRM fraction corresponding to T20LDL, thus suggesting that this fusion protein does not partition in lipid rafts. No specific band for T20 was found in any fraction of HEK-293CD4 control cells either.

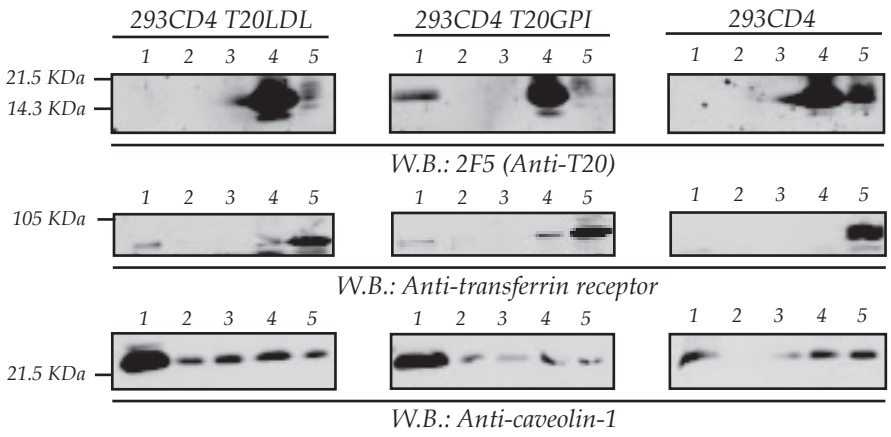


Figure 12. T20GPI partitions in detergent-resistant membranes.
T20LDL and T20GPI stable cell lines (HEK-293CD4) were lysed in the presence of Triton X-100 and ultracentrifuged in an Optiprep gradient. Six fractions were recovered from the ultracentrifugation, and fractions 1 and 2 were pooled together for western-blot analysis. Lane 1 corresponds to proteins found in detergent-resistant membranes, and lane 5 corresponds to the rest of solubilised proteins. Blotting was performed with 2F5 mAb, anti-human transferrin receptor, and anti-caveolin-1.

- ***Copatching experiments***

To clarify the results gathered from the DRM isolation, an immunofluorescence experiment was devised with the T20LDL- and T20GPI-expressing cell lines, in this case expressed in HEK-293T cells. This assay is called copatching, and relies on the crosslinking of fluorescently-labeled antibodies against raft markers that induces clustering of lipid rafts, which can be visualized by confocal microscopy. In the case at hand, the cholera toxin

subunit β biotin-coupled protein (CTx) was used to target the ganglioside GM1 found in lipid rafts, while the 2F5 antibody was used to detect T20 at the membrane. Fluorescently-labeled secondary antibodies were also added to the samples, to induce more crosslinkage and confer fluorescence to the cells (an anti-human IgG-Cy5 antibody in the case of T20 and streptavidin-Cy3 in the case of GM1); therefore, if the protein of interest is located in raft microdomains the colocalization signal will be purple, whereas if the proteins segregate in different patches in the membrane the fluorescent signals will be either red or blue. The images gathered, shown in Figure 13A, showed a mix of raft and non-raft localizations for both T20LDL and T20GPI samples, instead of a clear distribution of T20GPI in lipid rafts and T20LDL outside them. Consequently, a quantification of T20-GM1 signal colocalization was also performed, using the software provided by Image J. The resulting graphic is shown in Figure 13B, where it is evident that there is no significant difference in membrane localization between the two T20-based constructs. Additionally, the levels of colocalization of a GFPGPI construct with GM1 are similar to the ones gathered with the T20 constructs (the GFPGPI data derives from another experiment).

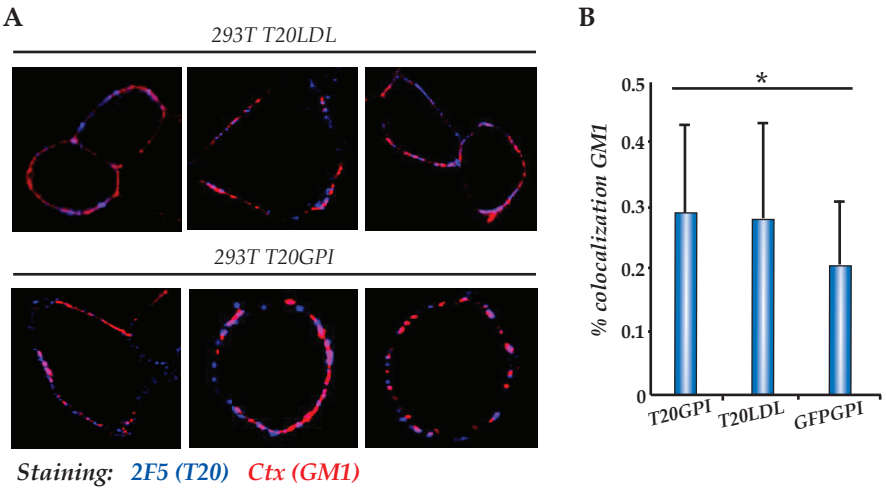


Figure 13. Both T20LDL and T20GPI show the same pattern of colocalization with GM1.
(A) T20LDL and T20GPI stable cell lines (HEK-293T) were stained using 2F5 mAb and CTx, as indicated. Patching was induced with fluorescently-labeled secondary antibodies (T20 is shown in blue and GM1 in red). Colocalization (purple) was analysed by confocal microscopy. (B) Colocalization values were obtained for each construct using Image J software. GFPGPI values are shown as comparison and were obtained from the experiment described in section Immunofluorescence (copatching) experiments for visualization of raft-associated proteins from MATERIALS AND METHODS. Bars indicate the average + SD (n=24 for T20GPI, n=12 for T20LDL and n=14 for GFPGPI). Statistical differences are indicated (two-tailed T-student test): *, P<0.05.

1.4 Effect of membrane-anchored T20LDL and T20GPI in Env-mediated cell-cell fusion

The next step taken to analyse the effect of anchoring T20 to the membrane of HIV-1 target cells was to perform fusion assays. These experiments consist in co-culturing two

different populations of cells: on one hand, effector cells that are infected with a vaccinia virus that induces the expression the gp160Env (IIIB) complex of HIV-1 at the membrane (to mimic the virus), and on the other hand target cells that possess the receptors necessary for HIV-1 entry (in this case CD4 and CXCR4) and are transfected with a reporter gene (luciferase). The reporter gene is under the control of a vaccinia virus promoter, and as such the luciferase gene will only be expressed if fusion has occurred between the two populations of cells. Besides the luciferase reporter gene, the target cells are also transfected with a renilla luciferase plasmid that will be expressed constitutively, to serve as an internal control for transfection. To analyse the effect of membrane-bound T20 in Env-mediated cell-cell fusion, the target cells for infection also express either T20LDL or T20GPI in a stable fashion (stable cell lines produced in HEK-293CD4 cells); the detection of a luciferase signal will be indicative of the occurrence of fusion between the two populations of cells, and will also provide evidence if T20 at the membrane is able to inhibit Env-mediated cell-cell fusion.

The results showed that T20GPI expression in target cells lowers the luciferase signal by 70% when compared to the positive control, i.e. HEK-293CD4 cells permissive to HIV-1 Env-mediated fusion (Figure 14). A more pronounced fusion inhibition was registered for cells expressing T20LDL, showing that this construct behaves in a very similar manner to T20GPI, although it was presumed to be localised outside lipid rafts. As a control, soluble T20 peptide was added externally to control target cells to check the sensitivity of the fusion process to T20 in our experimental setup. As expected, the soluble peptide had the ability to prevent fusion between the two populations of cells, in a dose-dependent manner (Figure 14). The CXCR4-antagonist AMD3100 was also added to HEK-293CD4 cells as another control of fusion inhibition, and almost no luciferase signal was detected, confirming that Env-induced cell-cell fusion is not an unspecific event but requires a functional CXCR4 coreceptor.

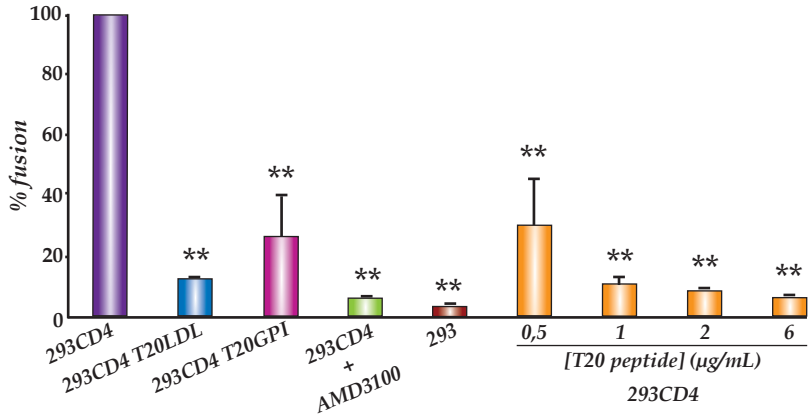


Figure 14. Membrane-bound T20LDL and T20GPI both inhibit Env-mediated cell-cell fusion.
T20LDL and T20GPI stable cell lines (HEK-293CD4) were cocultured with HEK-293 cells that express the HIV-1 Env complex at the membrane. Luciferase relative light units (RLUs), indicative of fusion, were obtained for HEK-293CD4 cells (positive control) and are shown as 100% fusion. All other values were normalised in accordance. Soluble T20 peptide (several concentrations) and AMD3100 were used as inhibitors of Env-mediated cell-cell fusion. Bars indicate average + SD (n=3-6). Statistical differences are indicated (two-tailed T-student test), with regard to control cells (293CD4): *, $P<0.05$; **, $P<0.01$.

1.5 Analysis of the entry of HIV-pseudotypes in T20LDL- and T20GPI-expressing cells

The next step taken was to analyse the ability of membrane-anchored T20 in preventing infection of target cells by free viruses. In order to do so, HIV-pseudotypes were generated and consequently used to infect cells that express T20 at the membrane. To produce the HIV-pseudotypes, HEK-293T cells were cotransfected with the pNL4.3*luc*R-E- vector (described in MATERIALS AND METHODS) along with a plasmid that codifies for a given envelope: either ADA (an R5 HIV strain), NL4.3 (an X4 HIV strain) or VSVG (control). These pseudotyped viruses are replication-deficient, because although they carry the machinery necessary for entry into permissive target cells, they lack the genes crucial for the assembly of new viral particles. Moreover, they possess the luciferase gene that will be expressed upon entry into the target cell; therefore, similarly to the fusion assay, luciferase activity will be used to monitor if the viruses are able to enter target cells and if T20 prevents that entry from taking place.

Once the viruses were produced and titrated for p24 concentration, equal amounts of ADA and NL4.3 (30 ng of p24 antigen) were used to infect T20-expressing cells; for VSVG a lower concentration was used (8 ng of p24 antigen), since the virus is more effective and the luciferase values obtained are several orders of magnitude higher than with the remaining viruses. Several infection assays were attempted using HEK-293CD4 T20LDL- and T20GPI-expressing cells as target cells, but the luciferase levels were too low for the results to be taken into consideration.

We therefore decided to use TZM-b1 cells, a HeLa-derived cell line that expresses high levels of CD4, CCR5 and CXCR4 and is commonly used in X4 and R5 infection experiments; since we did not have TZM-b1 cells that stably expressed T20 at the membrane, we performed infection experiments with TZM-b1 transiently expressing T20LDL or T20GPI constructs. The transfection efficiency is shown in Figure 15A, where around 23% of cells were positive for T20 expression in the membrane, both in the case of T20LDL and T20GPI. The infection assay (Figure 15B) indicated that the expression of T20LDL or T20GPI at the membrane of target cells scarcely affects the entry of ADA viruses (no infection was obtained with NL4.3 pseudotypes). However, the entry of VSVG viruses was inhibited by the membrane-anchored peptide. As a control, TZM-b1 cells were also incubated with three different concentrations of soluble T20 peptide (2, 5 and 10 μg/mL) 24h before infection (and renewed upon it), and it was confirmed that the peptide inhibited the entry of the ADA virus in a dose-dependent manner; furthermore, the cells incubated with soluble T20 were permissive for VSVG entry, as expected.

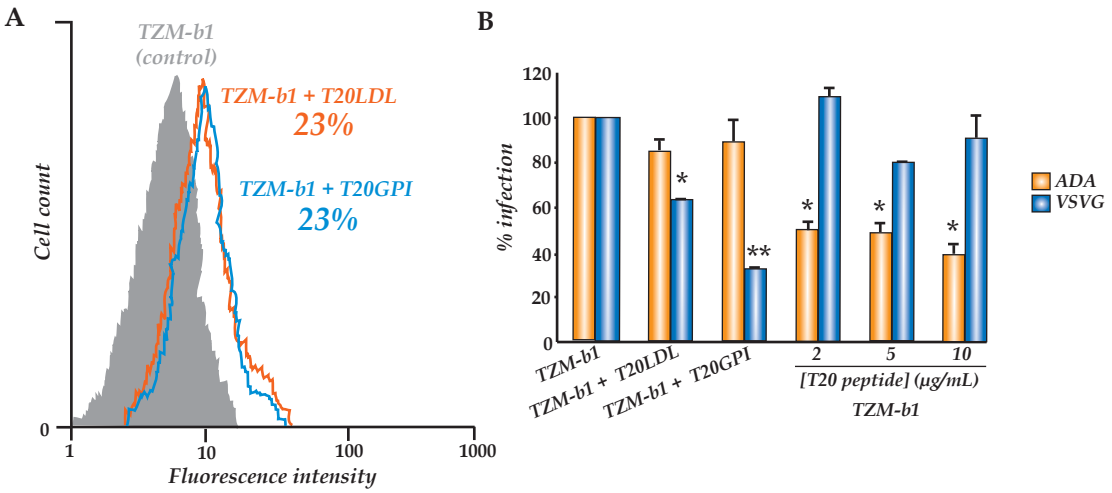


Figure 15. Transient expression of T20LDL and T20GPI at the membrane does not inhibit infection by free HIV-1 pseudotyped viruses.

(A) Flow cytometry analysis of TZM-b1 cells transiently expressing T20LDL or T20GPI. The percentages relative to positive staining for 2F5 mAb are indicated. (B) TZM-b1 cells transiently expressing T20LDL or T20GPI were infected with HIV-1 replication-deficient viruses pseudotyped with ADA or VSVG envelopes. Luciferase values, indicative of infection, were obtained for TZM-b1 cells (positive control) and are shown as 100% infection. All other values were normalised in accordance. Soluble T20 peptide (several concentrations) was used as inhibitor of HIV-1 infection. Bars indicate average + SD (n=2). Statistical differences are indicated (two-tailed T-student test), with regard to control cells (TZM-b1): *, P<0.05; **, P<0.01.

1.6 Analysis of the infectivity of HIV-pseudotypes produced in T20LDL- and T20GPI-expressing cells

We next investigated whether the incorporation of T20 in the envelope of emerging virions alters their infectivity; since newly-formed viral particles presumably exit the cell through lipid rafts (Nguyen and Hildreth, 2000), indeed incorporating characteristic cell proteins in their envelope (Arthur et al., 1992), we postulated that T20GPI will also be incorporated in the viral envelope upon exit. We therefore analysed if the presence of this T20 construct in the viral envelope would decrease the ability of those viral particles to engage a new target cell and initiate a new fusion process.

In order to test such hypothesis, HIV-pseudotypes were produced in T20-expressing cells. In this case, three viral envelopes were used in addition to the ones mentioned before: ADA 38E/42S (with a two-aminoacid substitution that confers resistance to T20-mediated inhibition), ADA 38A/42T (another two-aminoacid substitution conferring resistance to T20), and NL4.3 38E/42S (the same aminoacid substitution as ADA 38E/42S). The plasmids that codify for these envelopes were kindly provided by Rafael Delgado. The HIV-pseudotypes were produced in T20LDL- and T20GPI-expressing stable cell lines (HEK-293T), titrated for p24 concentration, and used in infection assays with TZM-b1 cells as targets of infection. Additionally, viruses produced in HEK-293T cells were also used as a positive control.

First, we analysed the susceptibility of the different pseudotypes produced in HEK-293T cells to T20-mediated inhibition (Figure 16A). The data obtained shows that, while the

ADA wildtype viruses are sensitive to T20-mediated inhibition, as would be expected, the NL4.3 are not. Furthermore, the two-aminoacid substitutions that were introduced in the ADA and NL4.3 envelopes to confer resistance to T20 behave differently: the 38E/42S combination seems to be relatively resistant to T20-mediated inhibition in ADA-pseudotyped envelopes, although 40% of inhibition was obtained at a T20 dosage of 10 µg/mL (Figure 16A). Given the resistance of the NL4.3 wildtype envelope to T20 inhibition, it is not surprising that the NL4.3 38E/42S mutant envelope would be totally resistant to the inhibitor at the dosage used. The introduction of the double 38A/42T mutation in the ADA envelope confers no resistance at all, in spite that these mutations were reported to prevent T20-mediated inhibition (Mink et al., 2005; Sista et al., 2004; Wei et al., 2002). As a control, VSVG viruses were also used in this assay and no change in infection with T20 incubation was registered, as would be expected.

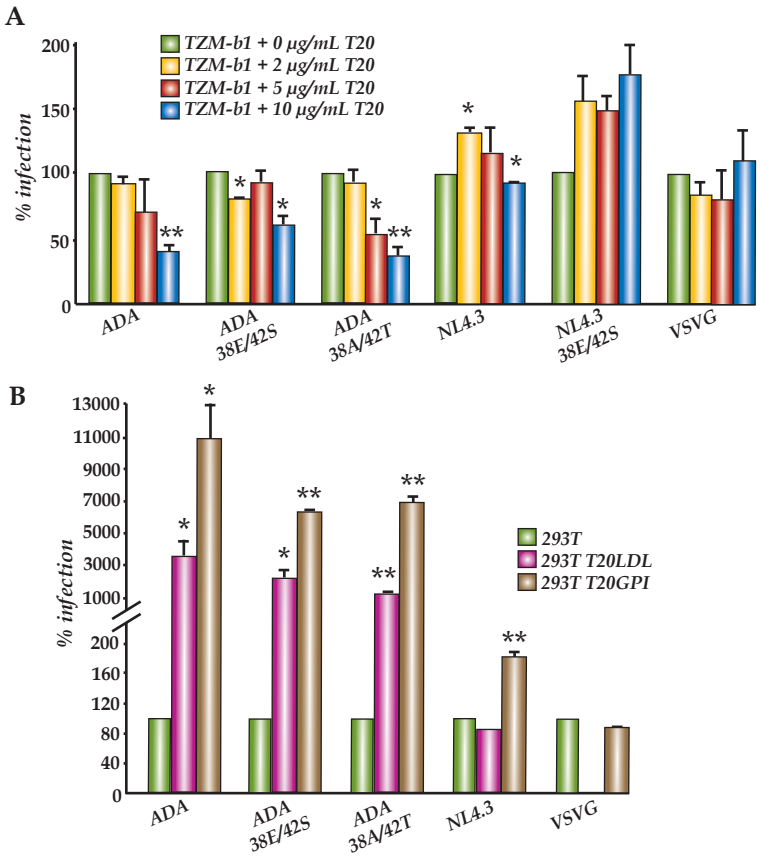


Figure 16. HIV-pseudotypes produced in T20LDL- and T20GPI-expressing cells are more infectious than viruses produced in control cells.

(A) HIV-1 replication-deficient viruses pseudotyped with ADA, ADA 38E/42S, ADA 38A/42T, NL4.3, NL4.3 38E/42S or VSVG envelopes were infected in TZM-b1 cells in presence of 0, 2, 5 or 10 µg/mL of soluble T20 peptide. Luciferase levels, indicative of infection, were obtained for TZM-b1 cells (control) and are shown as 100% infection. All other values were normalised in accordance. Bars indicate average + SD (n=2) (B) HIV-1 replication-deficient viruses pseudotyped with ADA, ADA 38E/42S, ADA 38A/42T, NL4.3 or VSVG envelopes were produced in T20LDL and T20GPI stable cell lines (HEK-293T) and used to infect TZM-b1 cells. Luciferase values, indicative of infection, were obtained for viruses produced in HEK-293T cells (positive control) and are shown as 100% infection. All other values were normalised in accordance. Bars indicate average + SD (n=2). Statistical differences are indicated (two-tailed T-student test), with regard to control cells (TZM-b1), in (A), or control viruses (produced in 293T cells) in (B): *, P<0.05; **, P<0.01.

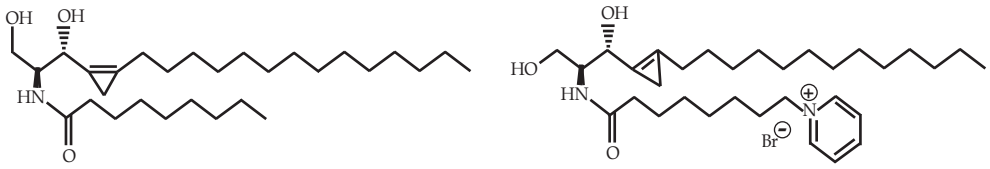
In Figure 16B are shown the different HIV-pseudotypes produced in either HEK-293T, T20LDL-expressing or T20GPI-expressing cells. The data indicates that all ADA strains used, when produced in cells that express T20 at the membrane, are several orders of magnitude more infectious than the same strains produced in control cells. Intriguingly, the same does not happen with the NL4.3 strain, which only showed a mild increase in infectivity when produced in T20GPI-expressing cells (Figure 16A). Once again, VSVG pseudotypes were used as a control and no difference in infectivity was detected between viruses produced in HEK-293T cells or T20-expressing cells.

Overexpression of either T20LDL or T20GPI at the membrane of target cells is able to inhibit X4 Env-mediated cell-cell fusion. Transient low expression of T20 at the membrane of target cells is not sufficient to inhibit entry of ADA pseudotypes. ADA pseudotypes produced in T20LDL- and T20GPI-expressing cells are more infectious than wildtype viruses.

2. Effect of the inhibition of dihydroceramide desaturase (DHCDase) in HIV-1 infection

Previous studies pointed out glycosphingolipids as key elements involved in the HIV-1 Env-mediated fusion process (Hammache et al., 1999; Harouse et al., 1991; Hug et al., 2000; Nehete et al., 2002; Puri et al., 2004) These evidences prompted us to study the effect on HIV-1 infection of a novel inhibitor in the sphingolipid pathway, GT11, developed by Gemma Fabriàs laboratory at the IIQAB in Barcelona (Triola et al., 2001). This compound is a ceramide analog that inhibits the enzyme dihydroceramide desaturase (DHCDase), responsible for the formation of a 4,5-*trans*-double bond in dihydroceramide that converts it to ceramide (Triola et al., 2004; Triola et al., 2001). Although GT11 does not prevent the formation of sphingolipids in the cell, the ones that are formed possess a dihydroceramide backbone instead of a ceramide one, which alters their biophysical and structural properties (Contreras et al., 2005). Therefore, treatment of cells with GT11 would induce an accumulation of saturated sphingolipids in lipid rafts; how this accumulation will affect lipid raft properties and function is not known, and we decided to study this subject using HIV-1 infection as a biological model. Our purpose is to better understand the implication of interfering with ceramide synthesis in cells.

Two different GT11 compounds are used in this approach: GT11 C8 (with a fatty acid chain composed by 8 carbons) and GT11pyr (a derivative of the original GT11 compound, with a pyrimidine ring) (Figure 17). The GT11pyr inhibitor was characterized more thoroughly and used in more assays because its pyrimidine ring confers it hydrosolubility, an advantage in *in vivo* studies. Both compounds have been tested in Dr. Fabriàs laboratory for inhibitory activity against DHCDase, and the IC_{50} values found for each one were 23 nM for GT11 C8 (Triola et al., 2004) and 27 μM for GT11pyr (unpublished data).



GT11 C8 *GT11pyr*

Figure 17. Chemical structure of GT11 C8 and GT11pyr.

2.1 Viability and cell cycle analyses in GT11-treated cells

To study if the compounds are well tolerated by cells, both GT11 C8 and GT11pyr were used in viability assays based on Trypan Blue dye exclusion method, and additionally the cells were stained for cell cycle analysis using propidium iodide. HEK-293, Jurkat and peripheral blood mononuclear cells (PBMCs) were used in these assays. According to the IC_{50} values reported for each inhibitor in *in vitro* assays, different concentration ranges were used, higher for GT11pyr than for GT11 C8.

In the case of GT11 C8, PBMCs were stimulated *in vitro* with PHA-L for 24h and further maintained in culture in the presence of IL-2. Cells were treated with several concentrations of GT11 C8 (0, 0.1, 0.5, 1 and 2 μM) for three consecutive days, counted everyday and stained with DNA Prep for cell cycle analysis. The data obtained from the experiment is shown in Figure 18A, where it is evident that GT11 C8 treatment is not detrimental to the cells, since there is no significant difference in cell number between cells incubated with the inhibitor and control cells. Noteworthy is the fact that, at least 24h after the start of the incubation with the compound, concentrations higher than 0,5 μM of GT11 C8 increased cell growth when compared with vehicle-treated cells. Staining with propidium iodide, however, showed no differences in all cell cycle phases for the last time point (Figure 18B). These results suggest that the compound is well tolerated by PBMCs, at least until 2 μM.

In the case of GT11pyr, PBMCs were stimulated *in vitro* with PHA-L for 24h and further maintained in culture in the presence of IL-2. Cells were treated with several concentrations of GT11pyr (0, 0.5, 1, 2, 5 or 10 μM) for three consecutive days, counted everyday and stained with DNA Prep for cell cycle analysis. The results are depicted in Figure 19, both for cell number (Figure 19A) and flow cytometry analysis of cell cycle (Figure 19B). The data shows that cell growth is affected by GT11pyr concentrations higher than 5 μM, and, accordingly, cell cycle measurements show that both 5 and 10 μM of GT11pyr slightly increase the number of apoptotic cells, for the last day assayed. Lower concentrations of the compound are less harmful to the cells. A similar analysis was performed with Jurkat cells, incubated with either 0, 1, 2, 5, 10 or 15 μM of GT11pyr. The results (Figure 20) show that GT11pyr concentrations higher than 5 μM also affect cell growth (Figure 20A), although lower concentrations are well tolerated by cells. Furthermore, cell cycle analysis (Figure 20B) confirms that the number of apoptotic cells increases proportionally with GT11pyr incubation.

In summary, these experiments indicate that the inhibitors GT11 C8 and GT11pyr have different toxicity on target cells. On one hand, GT11 C8 seems to be better tolerated by PBMCs, with little or no detrimental effect registered in these cells at the highest concentration used (2 µM). On the other hand, GT11pyr is more harmful, although concentrations lower than 5 µM are well tolerated by target cells.

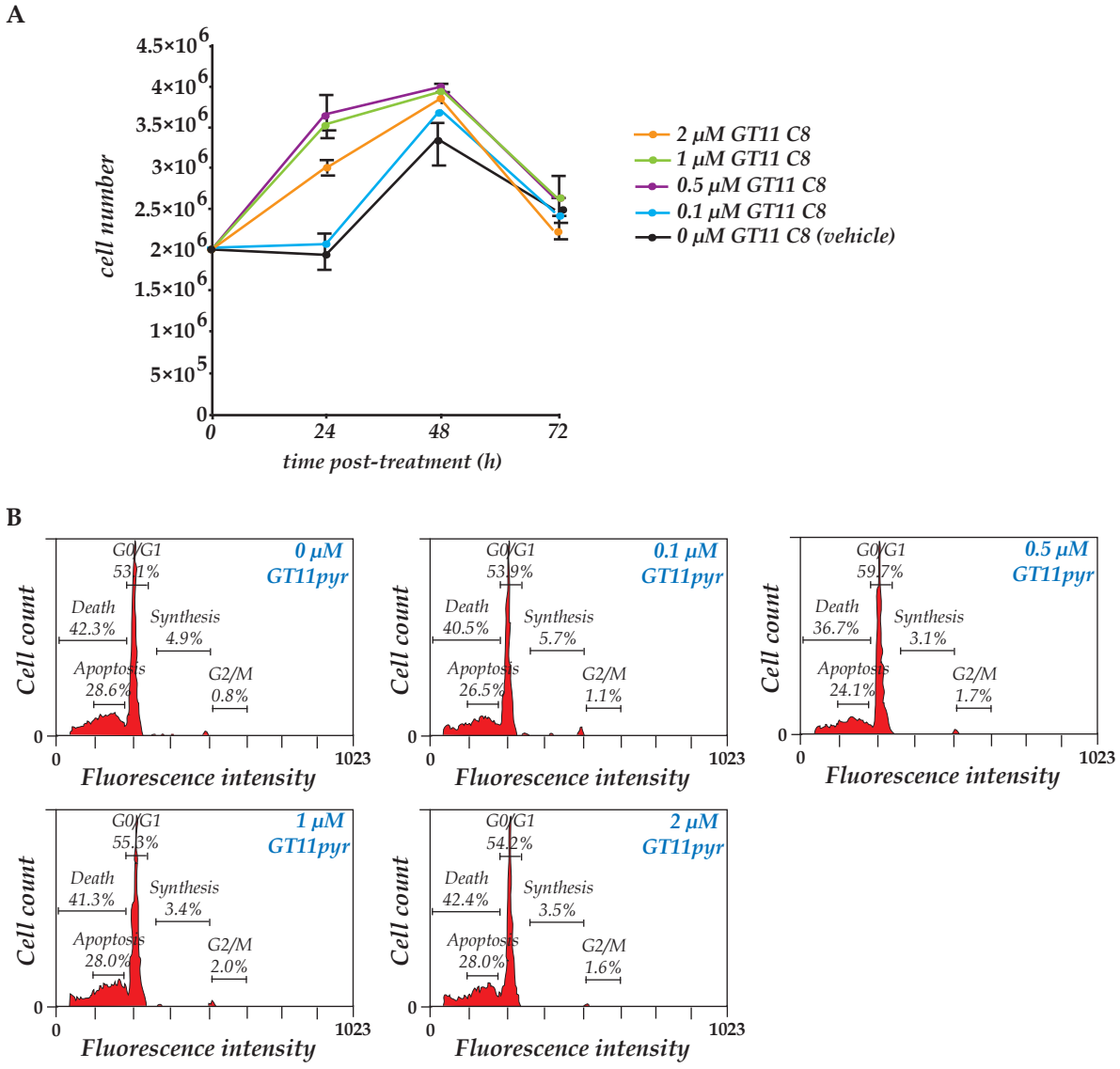


Figure 18. GT11 C8 does not affect the viability of PBMCs.
Activated PBMCs were incubated with 0, 0.1, 0.5, 1 or 2 µM of GT11 C8 for 3 consecutive days. (A) Cells were counted every day using Trypan Blue exclusion method. Shown is average ± SD (n=2). (B) Cells were stained with propidium iodide and cell cycle was analysed by flow cytometry. Shown is last day of incubation for each of the concentrations used. The percentage of cells in each cycle is indicated.

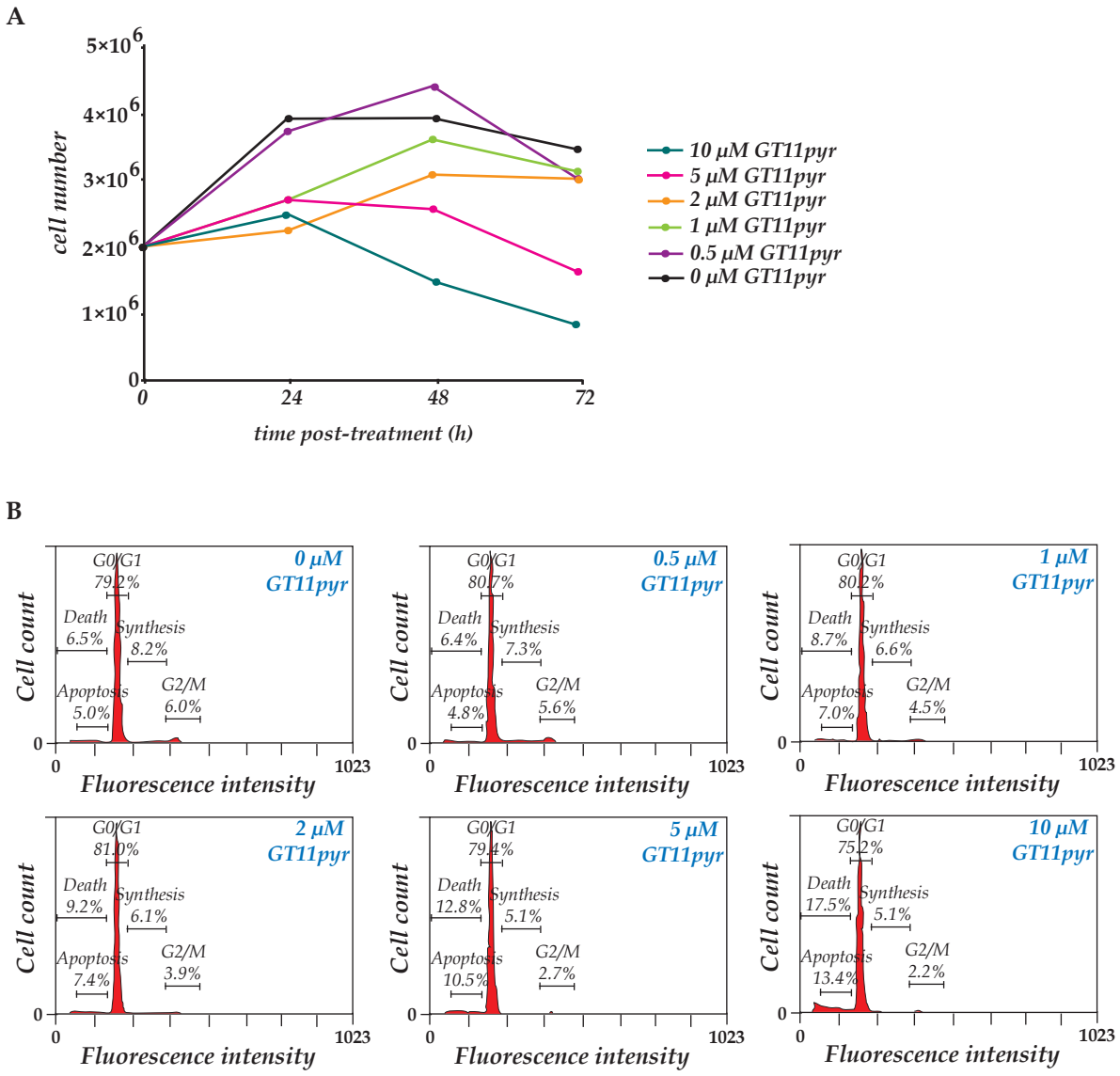


Figure 19. GT11pyr is toxic over 5 µM for PBMCs.

Activated PBMCs were incubated with 0, 0.5, 1, 2, 5 and 10 µM of GT11pyr for 3 consecutive days. (A) Cells were counted every day using Trypan Blue exclusion method. (B) Cells were stained with propidium iodide and cell cycle was analysed by flow cytometry. Shown is last day of incubation for each of the concentrations used. The percentage of cells in each cycle is indicated.

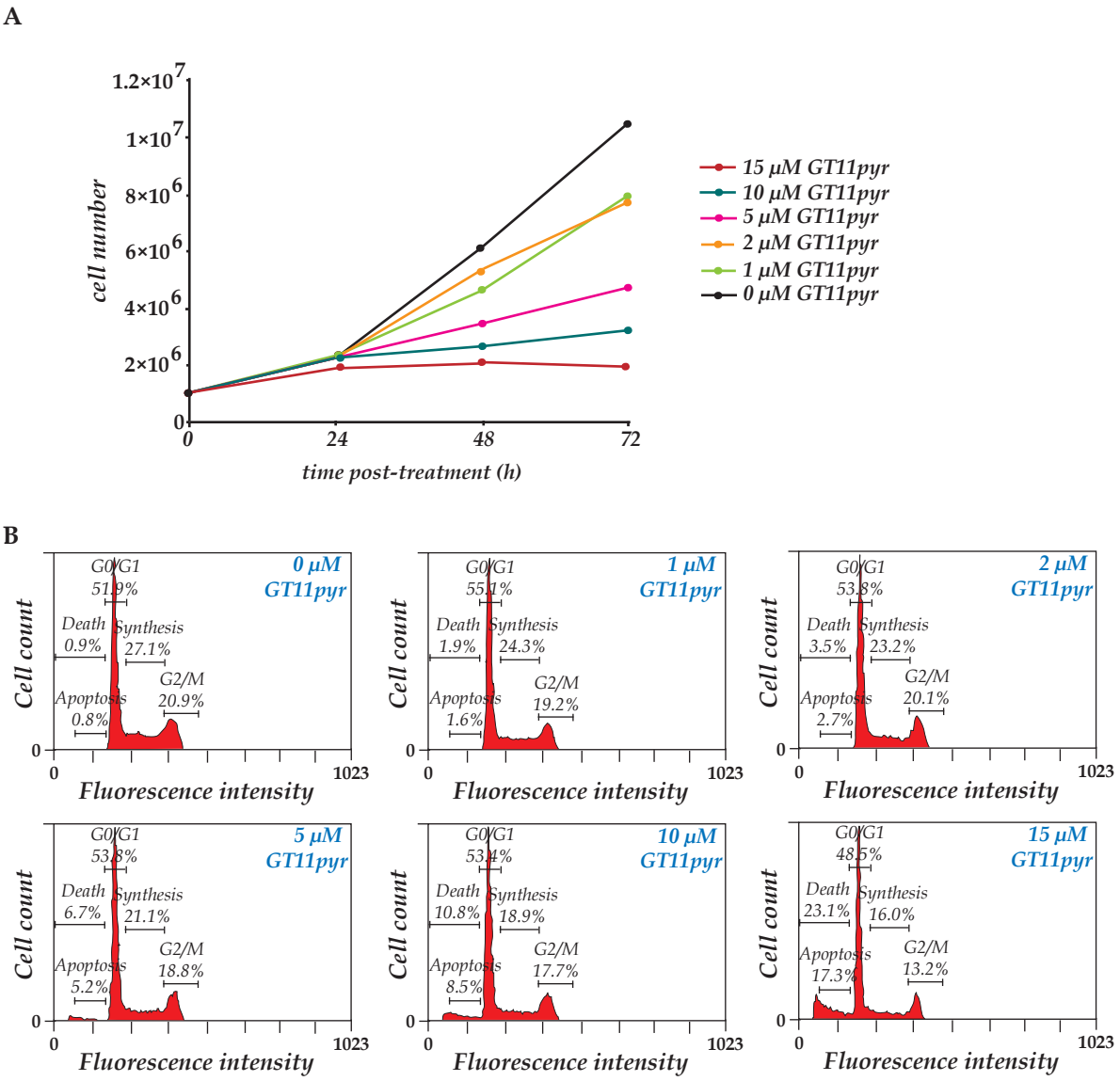


Figure 20. GT11pyr is toxic over 5 µM for Jurkat cells.

Jurkat cells were incubated with 0, 1, 2, 5, 10, and 15 µM of GT11pyr for 3 consecutive days. (A) Cells were counted every day using Trypan Blue exclusion method. (B) Cells were stained with propidium iodide and cell cycle was analysed by flow cytometry. Shown is last day of incubation for each of the concentrations used. The percentage of cells in each cycle is indicated.

2.2 Effect of GT11 on Env-mediated cell-cell fusion

In order to analyse whether GT11 has the ability to inhibit the entry of HIV-1 in the cell, Env-mediated cell-cell fusion assays were performed. The experiment is similar to the one described in section 2.1, but in this case target cells (HEK-293CD4) were incubated with 0, 0.01, 0.1, 0.5, 1, 5 or 10 µM of GT11 C8 for 24h before fusion, and renewed upon it. The results, depicted in Figure 21, show that there is a dose-dependent decrease in the luciferase signal when the cells are incubated with GT11 C8. Moreover, the estimated IC_{50} of GT11 C8 for this assay is 0.08 µM. This result indicates that this compound is able to inhibit Env-mediated cell-cell fusion at low concentrations.

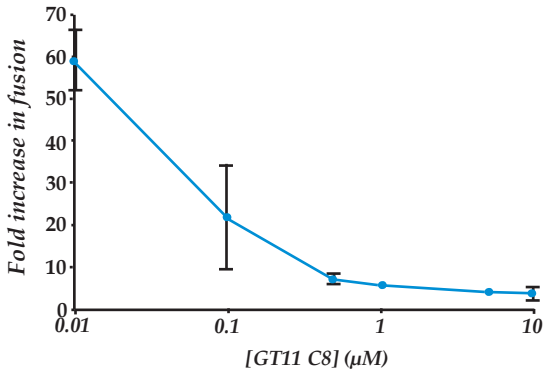


Figure 21. GT11 C8 inhibits Env-mediated cell-cell fusion in a dose-dependent manner. HEK-293CD4 cells were incubated with 0, 0.01, 0.1, 0.5, 1, 5 or 10 μM of GT11 C8 for 24h and cocultured with HEK-293 cells that express the HIV-1 Env complex at the membrane. Luciferase relative light units (RLUs), indicative of fusion, were normalised using the values obtained for HEK-293 cells (negative control) and are shown as fold increase in fusion. Shown is average ± SD (n=2).

2.3 Analysis of the entry of HIV-pseudotypes in GT11-treated cells

The next step taken was to analyse if GT11 is able to prevent infection of target cells by free viruses. Two different experiments were devised, in one case incubating target cells with high concentrations of GT11pyr for a short period of time, and in the other case using low concentrations of the compound over several days. In both cases, viral particles were produced by cotransfecting HEK-293T cells with the pNL4.3*luc*R-E- vector (described in MATERIALS AND METHODS) and a vector that codifies for a given viral envelope: ADA (R5 HIV strain), NL4.3 (X4 HIV strain) or VSVG (control). The pseudotyped viruses were titrated and equal amounts of p24 antigen were then used to infect GT11pyr-treated and control cells.

In the first approach mentioned, TZM-b1 cells were incubated with either 0, 15, 20 or 30 μM of GT11pyr for 24h before viral supernatants corresponding to 45 ng of p24 antigen were added to the cells for an additional 24h at 37ºC (GT11pyr incubation was maintained but not renewed). Moreover, citotoxicity assays indicated that the LD_{50} of the compound, for TZM-b1 cells, was 35 μM. The results, presented in Figure 22A, show that whereas the VSVG viruses are able to enter target cells at all concentrations tested, the entry of HIV-derived pseudotypes (both ADA and NL4.3) is significantly reduced. Concentrations of 15 μM upwards induce at least a 60% inhibition in viral entry for both ADA and NL4.3, but not for VSVG. This suggests that, by altering the lipid composition of lipid rafts using GT11pyr, the entry of HIV-based viral pseudotypes is specifically affected, when compared with a control virus (VSVG) that enters the cell through an endocytic pathway (Johannsdottir *et al.*, 2008).

In the second type of experiment performed, TZM-b1 cells were incubated with 0, 0.5, 1, 1.5 or 2 μM of GT11pyr for 36h, 60h or 84h before infection. Viral supernatants corresponding to 25 ng of p24 antigen were added to the cells for 24h at 37ºC (GT11pyr incubation was maintained but not renewed), and the results of the infection are shown in Figure 22B. As opposed to the results presented in Figure 22A, where at 24h post-treatment the effect in ADA and NL4.3 entry was already evident, when lower concentrations of GT11pyr are used the cells have to be in contact with the inhibitor for longer periods of time for an effect to be seen. In more detail, at 36h post-treatment there is no significant difference between ADA and VSVG entry in target cells, nor is there at 60h post-treatment. However, when the cells were incubated for 84h with

GT11pyr, the entry of ADA viruses is significantly reduced when compared to VSVG viruses, for all concentrations tested. This indicates that, when lower concentrations of GT11pyr are used (until 2 μM), longer periods of incubation are needed for an effect to be seen.

The infection results indicate that GT11pyr is able to specifically inhibit the entry of HIV-pseudotypes, in a dose-dependent manner. High concentrations of the compound produce a more drastic inhibition effect, whereas lower concentrations need longer incubation periods to inhibit the entry of these viruses. Furthermore, the entry of the VSVG virus, a non-lipid raft associated virus, is not affected by GT11pyr treatment.

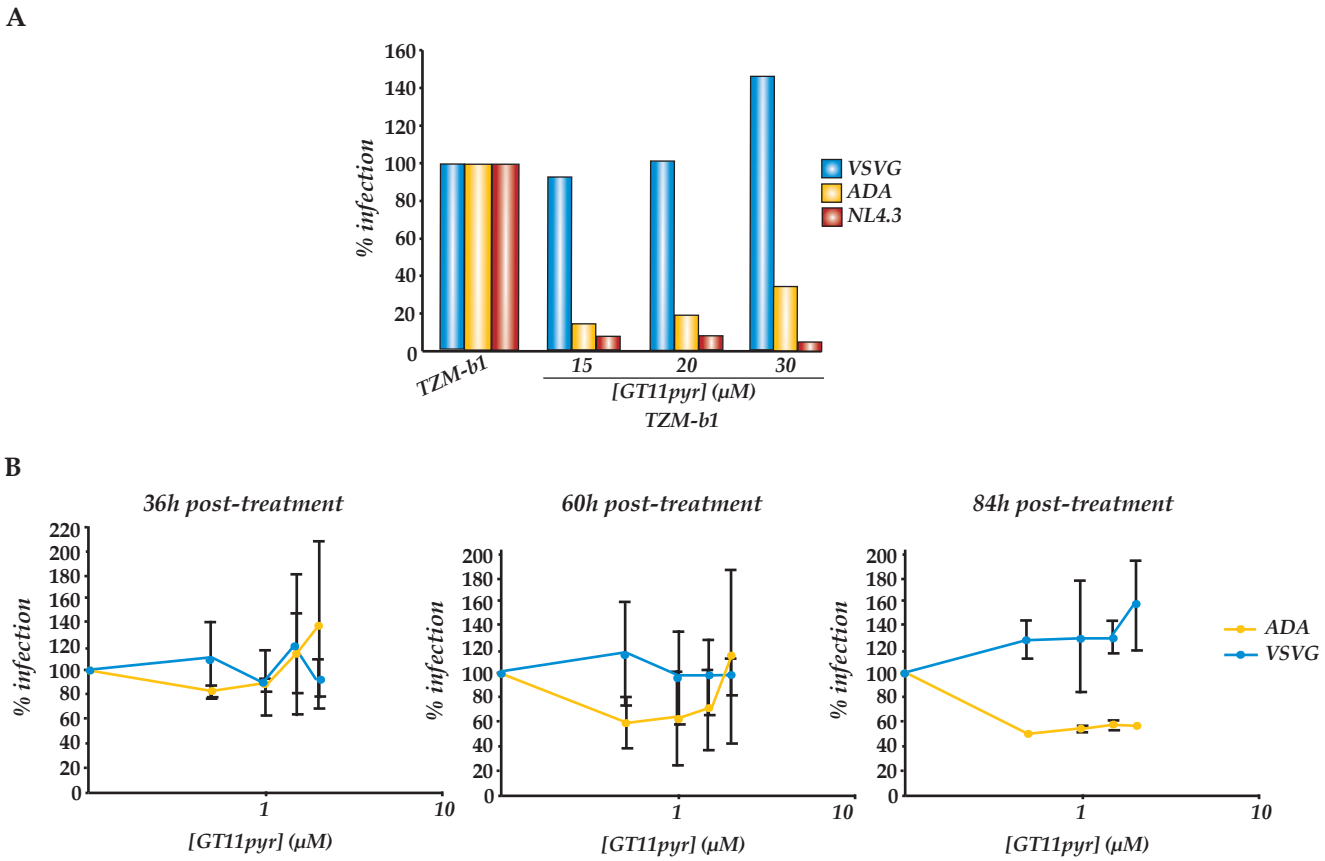


Figure 22. GT11pyr inhibits infection by free HIV-1 pseudotyped viruses.
(A) TZM-b1 cells incubated with 0, 15, 20 or 30 μM of GT11pyr for 24h were infected with HIV-1 replication-deficient viruses pseudotyped with ADA, NL4.3 or VSVG envelopes. Luciferase values, indicative of infection, were obtained for TZM-b1 cells (positive control) and are shown as 100% infection. All other values were normalised in accordance. (B) TZM-b1 cells incubated with 0, 0.5, 1, 1.5 or 2 μM of GT11pyr for 36h, 60h or 84h were infected with HIV-1 replication-deficient viruses pseudotyped with ADA or VSVG envelopes. Luciferase values, indicative of infection, were obtained for TZM-b1 cells (positive control) and are shown as 100% infection. All other values were normalised in accordance. Shown is average ± SD (n=2).

2.4 Effect of GT11 on T cell chemotaxis and activation

We next addressed the question of whether GT11 C8 and GT11pyr interfere with chemotaxis and T cell activation, two processes in which lipid rafts play a major role (Lin et al., 1999; Manes et al., 1999; Zhang et al., 1998b), at the concentrations at which these compounds inhibited HIV-1 infection.

- ***Chemotaxis assays***

An important characteristic of lymphocytes is the ability to migrate in response to a chemotactic gradient. To test whether blockade of DHCDase activity affects the chemotactic ability T cells, Jurkat cells and PBMCs were incubated with several concentrations of either GT11 C8 or GT11pyr and assayed for CXCR4-mediated chemotaxis in a modified Boyden chamber assay. In this type of experiment, cells are placed in the upper of two separate compartments communicated by a porous membrane, while in the lower compartment medium the appropriate chemokine is placed. After an incubation period of around 3-4 hours (depending on the cell line, explained in MATERIALS AND METHODS), the cells that have passed from the upper to the lower chamber are counted and represent the cells that have migrated in response to the chemotactic gradient.

The results, shown in Figure 23, indicate the amount of cells that have migrated in response to either complete medium or basal medium + CXCL12, when compared to basal medium alone, which represents background migration. In Figure 23A Jurkat cells were incubated with either 0.2 or 2 μM of GT11 C8. We found no differences in the chemotactic index when cells were incubated with vehicle or 0.2 μM of GT11 C8; surprinsingly, we observed a pronounced increase in the CXCL12-induced chemotaxis (more than 100 times over basal medium) when the cells were incubated with GT11 C8 at 2 μM. Interestingly, this increase was not observed when fetal calf serum (FCS) was used as chemoattractant, suggesting that GT11 C8 enhanced CXCL12-induced chemotaxis in a specific manner. We also found that GT11pyr did not substantially affect CXCL12- or FCS-induced chemotaxis of Jurkat cells in the 1 to 5 μM dosage range (Figure 23B). Nonetheless, we found again a specific amplification of the CXCL12-induced chemotaxis (10 times) when the cells were incubated with GT11pyr at 10 μM, a concentration that affected Jurkat cell growth (Figure 20A). As for PBMCs incubated with 0, 0.5, 1, 2 or 5 μM of GT11pyr, the results show a less pronounced increase in migration with GT11pyr incubation than the one registered with Jurkat cells (Figure 23C). Increasing concentrations of the inhibitor induce migration in response to CXCL12, but such migration only increases from 1.5 times over basal medium for control cells to 5.5 times over basal medium for cells incubated with 5 μM of GT11pyr.

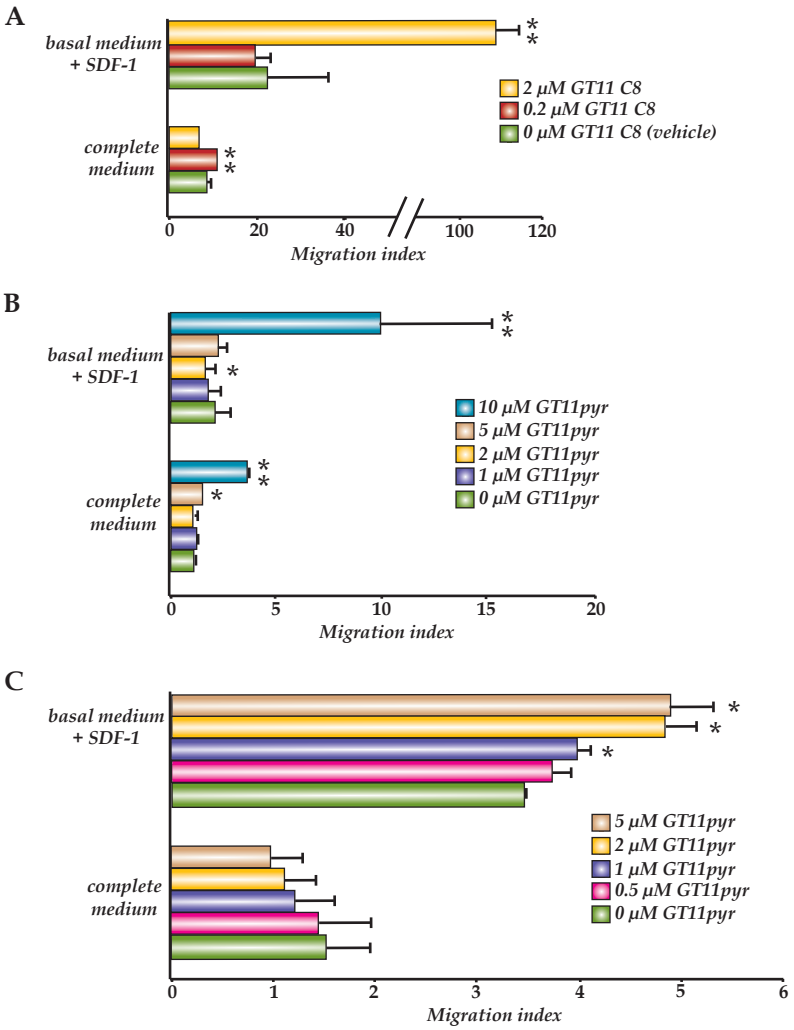


Figure 23. GT11 C8 and GT11pyr do not inhibit T cell chemotaxis.
(A) Jurkat cells were incubated with 0, 0.2 or 2 µM of GT11 C8 for 24h and placed in modified Boyden chambers to determine migratory response to the chemokine CXCL12. Migration index corresponds to the number of cells that migrated in response to fetal calf serum (FCS) or CXCL12 normalised with the corresponding number of cells that migrated in basal medium. Bars indicate average + SD (n=2). (B) Jurkat cells were incubated with 0, 1, 2, 5 or 10 µM of GT11pyr for 24h and the same type of analysis as in (A) was performed. Bars indicate average + SD (n=4). (C) PBMCs were incubated with 0, 0.5, 1, 2 or 5 µM of GT11pyr for 27h and the same type of analysis as in (A) was performed. Bars indicate average + SD (n=2). Statistical differences are indicated (two-tailed T-student test), with regard to control cells (0 µM of GT11pyr): *, P<0.05; **, P<0.01.

- ***IFNγ production***

Additionally, PBMCs were analised for the ability to become activated and produce IFNγ in the presence of GT11pyr. This is an important feature of T-lymphocytes and it is crucial to determine if incubation with the inhibitor does not impair the cells in their capacity to become activated in the presence of stimulatory factors. PBMCs were incubated with either 0, 0.5, 1, 2, 5, or 10 µM of GT11pyr, in complete medium without serum nor IL-2, in order to prevent unspecific activation. Following incubation with the inhibitor, the cells were stimulated with

anti-CD3 and anti-CD28 antibodies, still in the presence of the inhibitor, and the amount of IFNγ produced following stimulation was quantified by ELISA. The results, shown in Figure 24, indicate that incubation of the cells with low concentrations of GT11pyr is able to increase the production of IFNγ, although for higher concentrations (10 μM) the values revert to the control ones. More importantly, no inhibition or decrease of IFNγ output was detected with GT11pyr incubation.

In summary, our results indicate that GT11 C8 and GT11pyr, at concentrations interfering with HIV-1 infection, do not prevent other raft-associated processes in HIV-1 target cells, such as chemotaxis and TCR-mediated activation.

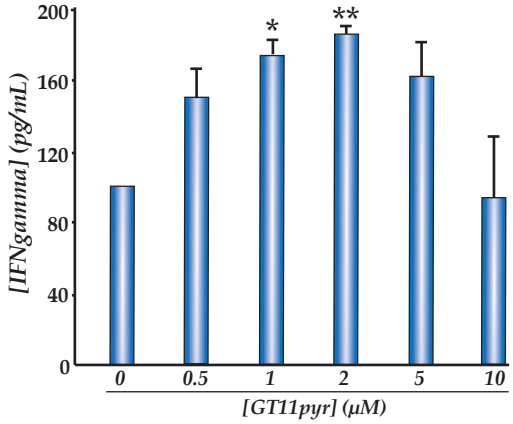


Figure 24. GT11pyr does not inhibit IFNγ production by PBMCs.
Non-stimulated PBMCs were incubated with 0, 0.5, 1, 2, 5, or 10 μM of GT11pyr for 36h at 37ºC. IFNγ was quantified in cell supernatants after activation with anti-CD3 and anti-CD28 antibodies for 12h. Bars indicate average + SD (n=2). Statistical differences are indicated (two-tailed T-student test), with regard to control cells (0 μM of GT11pyr): *, P<0.05; **, P<0.01.

2.5 Analysis of the lipid composition of detergent-resistant membranes (DRMs) in GT11-treated cells

We next analysed whether GT11 treatment alters the lipid composition of raft microdomains. DRMs were isolated from HEK-293CD4 cells (see section 1.3) pre-treated with GT11pyr at 15, 30 or 60 μM for 24h. In these conditions, GT11pyr treatment showed minor effects on HEK-293 viability, with the exception of the highest concentration of the compound (Figure 25A). The lipid composition of the DRMs was analysed in Dr. Fabriàs laboratory using High Performance Liquid Chromatography (HPLC) coupled to time of fly (TOF) mass spectrometry (HPLC-MS), and using C16 sphingomyelin as a probe, since this lipid is very abundant in lipid rafts. The resulting chromatograms from fractions that correspond to DRMs are shown in Figure 25B. It was found that GT11pyr treatment promoted a decrease in the relative amount of sphingomyelin (SM), which paralleled an increase in the amount of dihydrosphingomyelin (dhSM) in the DRM fraction. This change in the dhSM/SM ratio was dependent of the GT11pyr dosage used, reaching a maximum of 30% at a GT11pyr concentration of 30 μM (Figure 25C). The highest concentration of GT11pyr tested (60 μM) did not increase the dhSM/SM ratio, most likely because the compound is toxic for cells at this dosage. These results are consistent with a GT11pyr-induced inhibition of dihydroceramide desaturase *in vivo*, thus causing the accumulation of dihydrosphingolipids in lipid rafts.

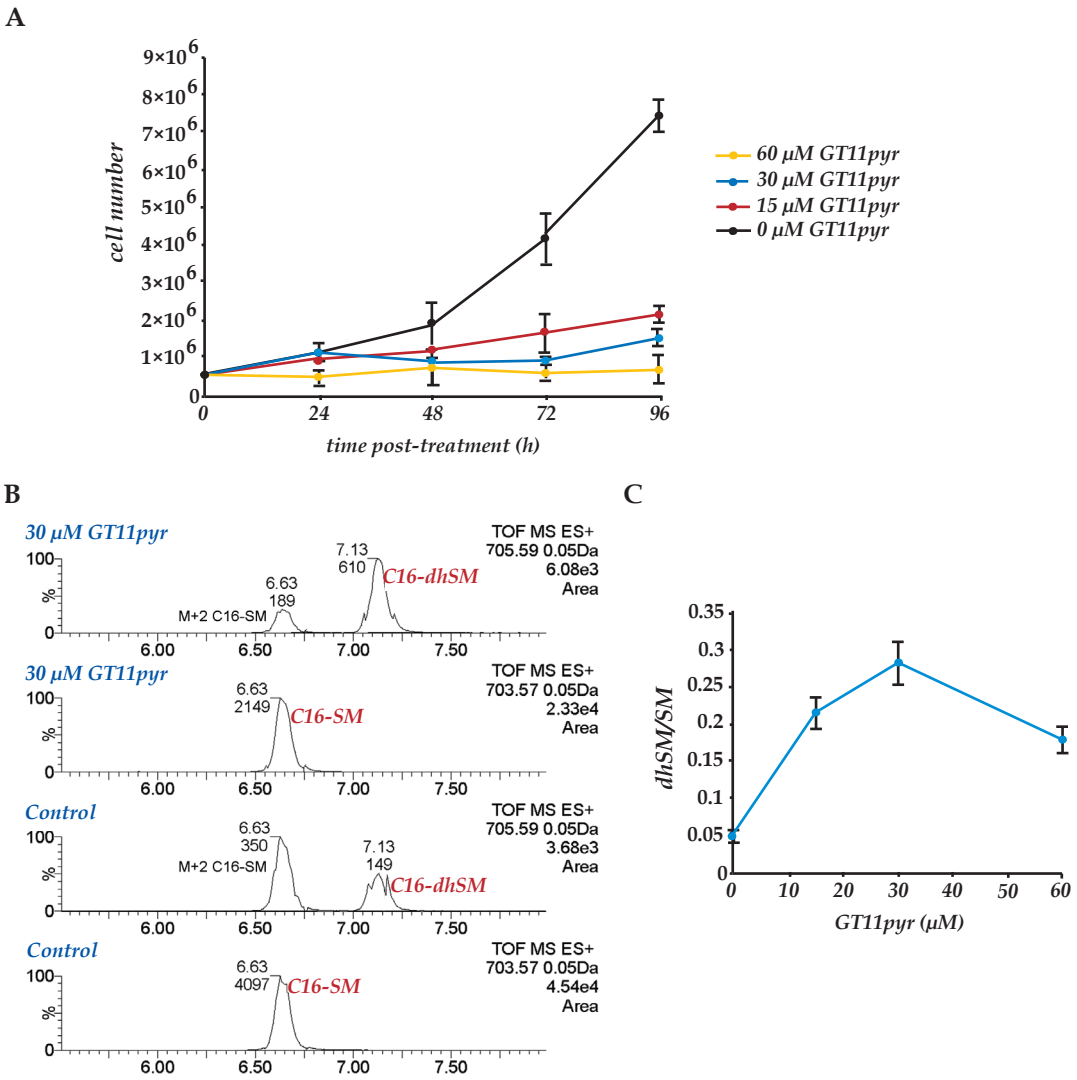


Figure 25. GT11pyr induces a replacement of SM for dhSM in lipid rafts.
(A) HEK-293 cells were incubated with 0, 15, 30 or 60 µM of GT11pyr for 3 consecutive days. Cells were counted every day using Trypan Blue exclusion method. Shown is average ± SD (n=2). (B) HEK-293CD4 cells were incubated with 0, 15, 30 or 60 µM of GT11pyr for 24h, lysed in the presence of Triton X-100 and ultracentrifuged in an Optiprep gradient. Six fractions were recovered from the ultracentrifugation and analysed by HPLC-MS. Chromatograms relative to the first fraction (DRMs) are shown for the control and 30 µM samples. (C) dhSM and SM were quantified for each sample and the dhSM/SM ratio is indicated for each concentration assayed. Shown is average ± SD (n=2).

2.6 Analysis of the protein composition in lipid rafts

We next analysed whether GT11-induced changes in the lipid composition of lipid rafts affected the preferential partitioning of specific membrane proteins into these microdomains.

- ***DRM isolation***

The same fractions from the ultracentrifugation used for the analysis of the lipid

composition described in section 2.5 were also employed to analyse the partitioning of the CD4 receptor by western-blot, as well as controls for DRM-associated and DRM-excluded proteins. The results are depicted in Figure 26, showing the comparison between the concentration of GT11pyr that promoted the maximal dhSM/SM ratio (30 µM; Figure 25C), and control cells. No difference in CD4 localization is detected between control cells and cells incubated with the inhibitor. As previously reported, the CD4 receptor was distributed between the DRM fraction (1st lane) and the fraction corresponding to solubilized membrane proteins (5th lane); this distribution was not altered by GT11pyr incubation (Figure 26A and B). The unique partitioning of caveolin and of the transferrin receptor, used as controls for DRM-associated and DRM-excluded membrane proteins, indicated the quality of the gradients.

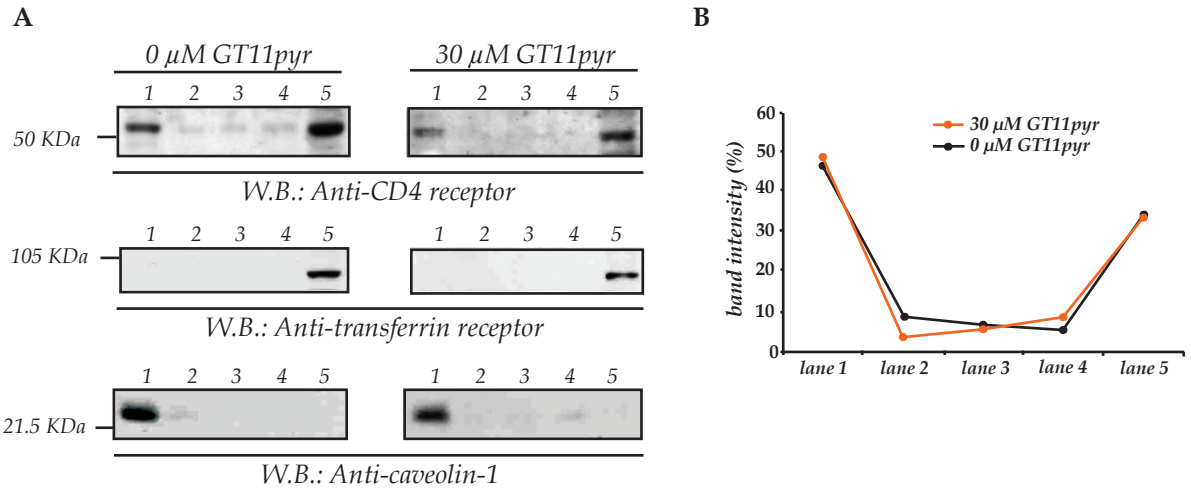


Figure 26. GT11pyr does not affect CD4 partitioning in detergent-resistant membranes.
(A) HEK-293CD4 cells were incubated with 0, 15, 30 or 60 µM of GT11pyr for 24h, lysed in the presence of Triton X-100 and ultracentrifuged in an Optiprep gradient. Six fractions were recovered from the ultracentrifugation, and fractions 1 and 2 were pooled together for western-blot analysis. Lane 1 corresponds to proteins found in detergent-resistant membranes, and lane 5 corresponds to the rest of solubilised proteins. Blotting was performed with anti-CD4 receptor, anti-human transferrin receptor, and anti-caveolin-1. (B) In the case of the CD4 receptor, band quantification was performed for each fraction.

- ***Copatching experiments***

To test if GT11pyr incubation is able to affect the membrane localization of raft-associated proteins, HEK-293CD4 cells were incubated with either 0 or 60 µM of GT11pyr and an immunofluorescence assay was performed, similar to the one detailed in section 1.**2**. The cells were also transfected with a vector encoding for a GFPGPI construct, as a positive control for raft-associated proteins. After a 24h-incubation period with the inhibitor, both treated and non-treated cells (control) and GFPGPI-transfected and non-transfected cells were placed in fibronectin-coated chambers for an additional 4h, in the presence of GT11pyr. Afterwards, primary antibodies against GM1, CD4, CXCR4 or GFP were added to the samples, followed by secondary fluorescently-labeled antibodies to induce patching of these proteins

at the membrane of the cells. Once again, CTx was used to label lipid raft-based GM1, and the colocalization of the aforementioned proteins with the fluorescent signal detected for GM1 gave an indication of whether the proteins were found in lipid rafts or not.

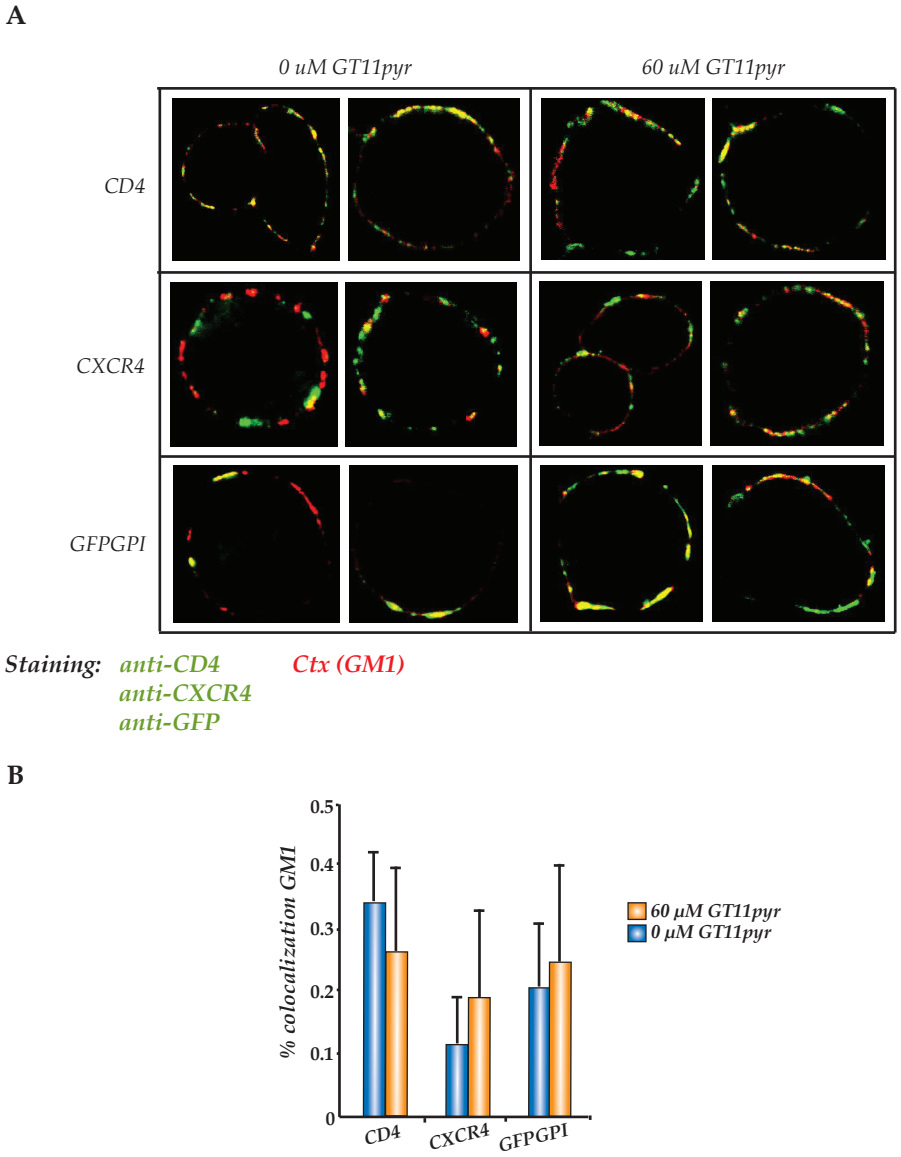


Figure 27. GT11pyr does not alter CD4, CXCR4 and GFPGPI colocalization with GM1.
(A) HEK-293CD4 cells were transfected with GFPGPI and incubated with 0 or 60 µM of GT11pyr for 24h. Cells were stained using anti-CD4, anti-CXCR4, anti-GFP and CTx, as indicated. Patching was induced with fluorescently-labeled secondary antibodies (CD4, CXCR4 and GFPGPI are shown in green and GM1 in red). Colocalization (yellow) was analysed by confocal microscopy. (B) Colocalization values were obtained for each staining using Image J software. Bars indicate average + SD (n=8-14 depending on the sample).

Figure 27 shows an assortment of confocal images for each of the conditions used (Figure 27A), representative of the total of images processed (in red is always shown GM1, and in green either CD4, CXCR4 or GFPGPI, as indicated), and a graphic with the quantification of CD4-GM1, CXCR4-GM1 and GFPGPI-GM1 signal colocalization (Figure 27B), in order to clarify the situation of each protein regarding lipid rafts. When comparing the colocalization of each protein with GM1 in basal conditions (without GT11pyr) and in cells incubated with 60 μM of GT11pyr, the percentages indicate that there was no significant difference for neither the CD4 receptor, the CXCR4 receptor or the GFPGPI protein (Figure 27B). This suggests that treatment with 60 μM of GT11pyr does not alter the localization of these raft-associated proteins, which is consistent with the DRM protein analysis showed above (Figure 26). Interestingly, the colocalization values obtained for CXCR4 are lower than the ones obtained for the remaining proteins, suggesting that the partition coefficient of this receptor into lipid rafts is lower than that of the CD4 receptor, in non-stimulated conditions. This agrees with previous reports showing that CXCR4 partitioning into lipid rafts changes upon stimulation (Manes et al., 2000).

2.7 Analysis of the effect of dihydrosphingomyelin in lipid membranes

The results detailed above showed that GT11pyr induced changes in the lipid composition of the cell membrane, but did not affected the association of HIV-1 receptors to lipid rafts. To better understand the biological implication of changing the composition of lipid rafts from SM to dhSM, Felix Goñi laboratory at the UPV/EHV in Bilbao studied the biophysical properties of dhSM in lipid bilayers of different composition.

- ***Negative curvature structures***

Since the occurrence of fusion between the viral and the cellular membranes requires the formation of a negative curvature structure, the effect of dhSM on the lamellar-inverted hexagonal phase transition of dielaidoylphosphatidylethanolamine (DEPE) model membranes was assessed. In Figure 28A are shown the thermograms relative to the lamellar-inverted hexagonal transitions of different DEPE-dhSM mixtures, and it is evident that with increasing concentrations of dhSM the transition temperature rises. This suggests that the presence of dhSM difficults the formation of negative curvature structures. In Figure 28B the same analysis was performed but with more complex mixtures of lipids: DEPE-5%dhSM, DEPE-5%SM, DEPE-5%dhSM:cholesterol(Ch) (1:1), DEPE-5%SM:Ch (1:1) and DEPE-5%SM:dhSM:Ch (0.7:0.3:1). The results show that both SM and dhSM induced a roughly similar increase in the transition temperatures in DEPE bilayers. Furthermore, the replacement of SM for dhSM did not change the transition temperature, suggesting that the substitution of one lipid by the other does not affect the formation of negative curvature structures.

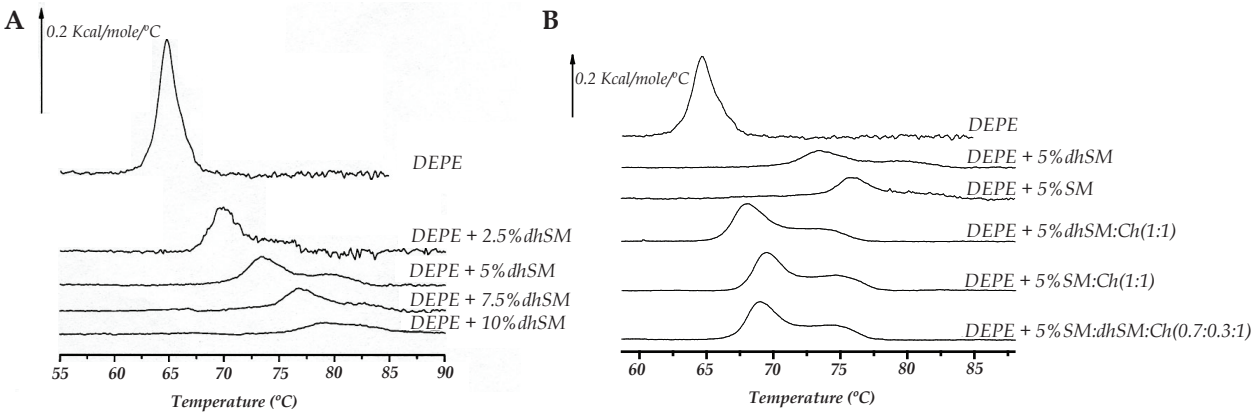


Figure 28. Replacement of SM for dhSM does not affect the formation of negative curvature structures in lipid bilayers.
(A) DSC thermograms relative to lamellar-inverted hexagonal transitions of DEPE and DEPE-dhSM mixtures in aqueous solution. The concentration of DEPE is constant (4 mM). (B) DSC thermograms relative to lamellar-inverted hexagonal transitions of different mixtures of DEPE, dhSM, SM and Ch in aqueous solution. The concentration of DEPE is constant (4 mM).

- ***Membrane rigidity***

Another possible effect of modifying the lipid composition of the membrane is the alteration of membrane rigidity. In order to test it, the generalised polarization of Laurdan-labeled membranes was used to assess membrane fluidity in SM:Ch (1:1), SM:dhSM:Ch (0.7:0.3:1), SM:dhSM:Ch (0.5:0.5:1) and dhSM:Ch (1:1) mixtures. The results, shown in Figure 29, indicate a progressive increase in generalised polarisation when dhSM is present at a higher concentration in the membranes, suggesting an increase in membrane rigidity. Of note, this effect on membrane rigidity was observed by increasing dhSM only 30%, which is the same dhSM replacement measured by HPLC-MS in cells treated with GT11pyr at 30 μM for 24h (Figure 25B). This is of particular interest to our work, since an increase in membrane rigidity could difficult the insertion of the fusion peptide in the target cell and additionally cholesterol in the membrane would be less accessible to recognition by the gp41 CRAC motif (Vincent et al., 2002).

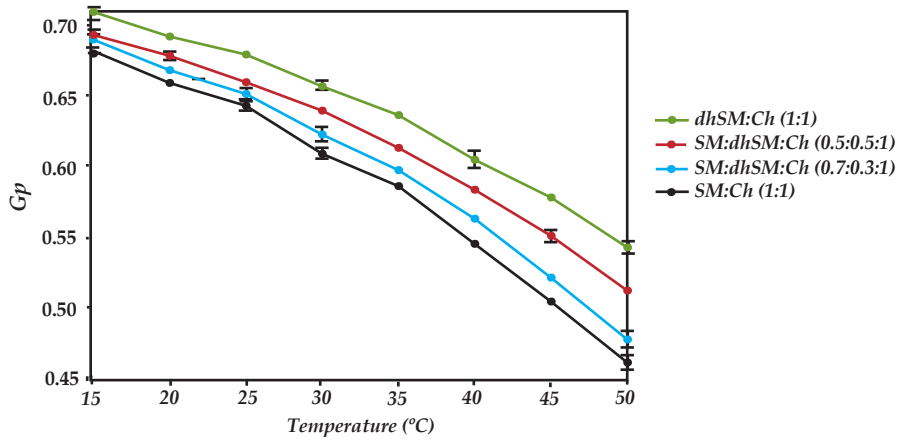


Figure 29. Replacement of SM for dhSM increases membrane rigidity in lipid bilayers.
Generalised polarization of Laurdan-labeled membranes with different SM, dhSM and Ch mixtures.

The next step was to use more complex mixtures of lipids in giant unilamellar vesicles (GUVs), to better understand the effect of dhSM in more physiological conditions. GUVs of PC:PE:SM:Ch (1:1:1:1) or PC:PE:dhSM:Ch (1:1:1:1) composition were studied with confocal microscopy using two fluorescent probes, DiI and NBD-Cer. DiI is only able to incorporate itself in fluid phases, whereas NBD-Cer can insert itself in both fluid and liquid ordered phases, although it has difficulty incorporating itself in rigid structures; by analysing the different incorporation of each probe we can assess whether the substitution of SM by dhSM has an effect on phase formation in GUVs. As seen in Figure 30A, GUVs formed by PC:PE:SM:Ch present two distinct phases, one fluid (stained with DiI and NBD-Cer) and another liquid ordered (stained with only NBD-Cer). However, when SM is replaced by dhSM, NBD-Cer still stained the same regions as DiI (fluid phases), but there was less incorporation of NBD-Cer in the remaining areas (corresponding to l_o membranes) (Figure 30B). This suggests that the combination of dhSM with cholesterol has formed a more rigid phase than the previously SM-Ch liquid ordered regions in PC:PE:SM:Ch GUVs. This agrees with the results obtained with the Laurdan-labeled membranes presented above, and suggests that GT11 treatment may affect HIV-1 infection by increasing membrane rigidity, which in turn may impair the insertion of the gp41 fusion peptide in the host membrane.

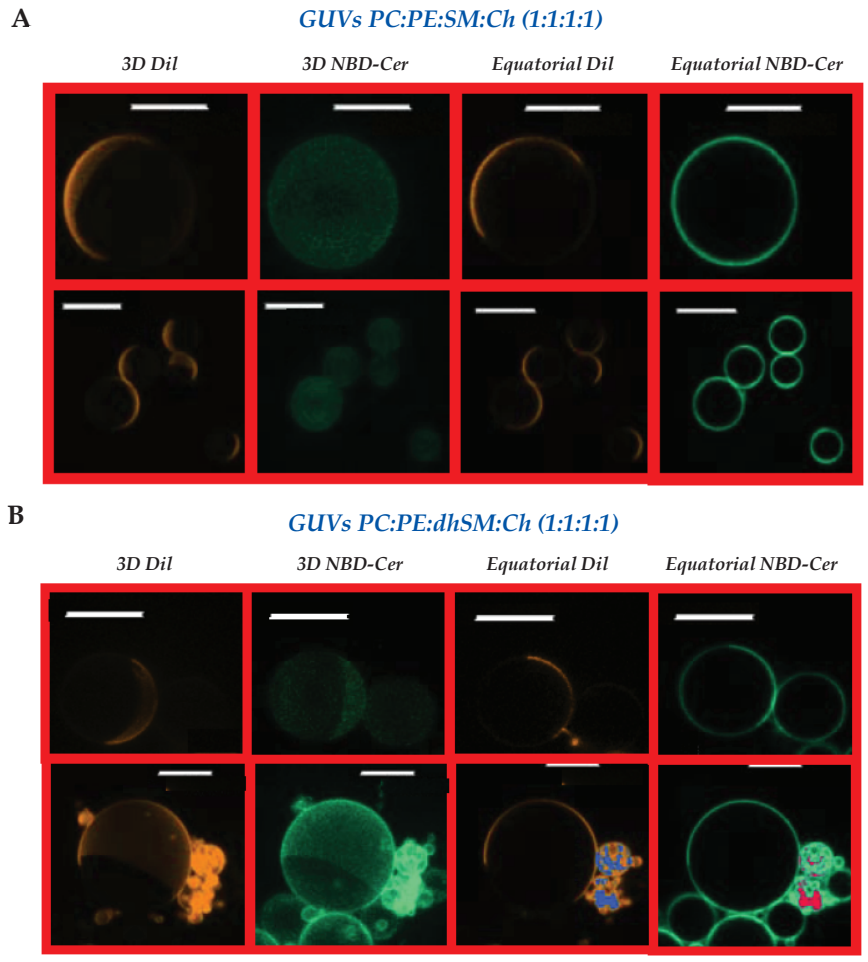


Figure 30. Replacement of SM for dhSM increases the rigidity of liquid-ordered phases in giant unilamellar vesicles.
(A) Giant unilamellar vesicles (GUVs) of PC:PE:SM:Ch (1:1:1:1) stained with DiI and NBD-Cer. (B) GUVs of PC:PE:dhSM:Ch (1:1:1:1) stained with DiI and NBD-Cer.

2.8 Analysis of the infectivity of HIV-pseudotypes produced in GT11-incubated cells

Lipid rafts not only are important in the entry of HIV-1 in the cell, but also in their exit from the infected cell, since the budding of newly-formed viral particles takes place in these microdomains in the membrane (Nguyen and Hildreth, 2000). To assess whether the modification of the lipid composition and membrane rigidity could alter the infectivity of newly-formed HIV-1 virions, ADA pseudotypes were produced in cells incubated with either 0 or 30 μM of GT11pyr (ADA-GT11 viruses). Furthermore, to ensure that GT11pyr present in the medium when recollecting the viruses does not affect the target cells when used in infection assays, the viral particles were ultracentrifuged and resuspended in fresh medium. Viral supernatants corresponding to 35 ng of p24 antigen were added to the TZM-b1 cells and the results from the infection are presented in Figure 31. We found that ADA-GT11 pseudotypes infected twice more effectively target cells than ADA viruses produced in non-treated cells. Unfortunately, no VSVG viruses were produced in the same conditions for comparison. Although a characterization of the SM/dhSM composition of the ADA-GT11 virions has not been performed, it is reasonable to envision that – as accounted for the recipient cells – their lipid composition would also be altered. Assuming this hypothesis, our results suggest that an enrichment of dhSM in the viral envelope would favour, rather than prevent, free virus-cell infection.

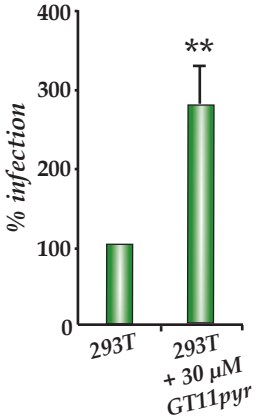


Figure 31. HIV-1 pseudotyped viruses produced in GT11-treated cells are more infectious than viruses produced in control cells.
HIV-1 replication-deficient viruses pseudotyped with ADA envelope were produced in HEK-293T cells incubated with 0 or 30 μM of GT11pyr for 36h, and used to infect TZM-b1 cells. Luciferase values, indicative of infection, were obtained for viruses produced in HEK-293T cells (positive control) and are shown as 100% infection. The value obtained for viruses produced in the presence of GT11pyr was normalised in accordance. Bars indicate average + SD (n=3). Statistical differences are indicated (two-tailed T-student test), with regard to control viruses (produced in 293T cells): **, P<0.01.

GT11 treatment inhibits both Env-mediated cell-cell fusion and infection by free HIV-pseudotypes. Incubation of target cells with GT11 induces a replacement of SM for dhSM in lipid rafts, but does not affect T cell associated functions nor the localization of raft-associated proteins. Higher dhSM content in membranes increases membrane rigidity. ADA pseudotypes produced in GT11-treated cells are more infectious than control viruses.

DISCUSSION

The entry of HIV-1 in the target cell is a complex process that entails several coordinated interactions between viral and host elements. First, the viral gp120 glycoprotein needs to interact sequentially with the CD4 receptor and a chemokine coreceptor (Dalgleish et al., 1984; Deng et al., 1996; Feng et al., 1996; Landau et al., 1988; Thali et al., 1993). Subsequently, the unfolding of the viral gp41 glycoprotein enables the insertion of the fusion peptides in the membrane of the cell, causing the formation of a fusion pore (Markosyan et al., 2003; Weissenhorn et al., 1997). Although the process of entry essentially follows these well-defined steps, many other factors confer complexity to the process and are crucial for a successful infection (Dimitrov, 2000). Fusion between viral and host cell membranes is a cooperative process that requires the sum of many CD4-gp120-coreceptor complexes. In fact, it is estimated that four to six CCR5 receptors (Kuhmann et al., 2000), a number of CD4 molecules (Kuhmann et al., 2000; Layne et al., 1990) and three to six Env trimmers are needed to form a fusion pore. In order for the formation of the entry complex, it is necessary the recruitment of the chemokine coreceptors in a CD4-gp120 interaction dependent manner, which has been described in several studies (Lapham et al., 1996). The clustering required for HIV-1 infection is not a passive diffusion process, it depends on multiple intermolecular interactions on the cell surface modulated by rapid cytoskeleton reorganization (Gouin et al., 2005; Iyengar et al., 1998; Jolly et al., 2004; Nguyen et al., 2005) and, at the plasma membrane, by the assembly of specialized lipid domains enriched in cholesterol, called lipid rafts (Hug et al., 2000; Liao et al., 2001; Manes et al., 2000; Popik et al., 2002; Viard et al., 2002).

Lipid rafts are specific microdomains in the cell membrane mainly composed by sphingolipids, cholesterol and saturated phospholipids (Brown and London, 2000; Simons and Ikonen, 1997). Due to their composition, these microdomains pack more tightly than the rest of the membrane, composed mostly by unsaturated phospholipids, and act as platforms that float in a fluid surrounding (Harder and Simons, 1997). Importantly, several proteins segregate preferentially to lipid rafts, such as the receptors necessary for the entry of HIV-1 in the cell. Not only is the specific localization of these receptors in these domains essential for viral entry (Del Real et al., 2002), but also the clustering capacity of lipid rafts has to be intact for the formation of a trimeric gp120-CD4-coreceptor complex (Brown and Rose, 1992; Manes et al., 2000; Nguyen et al., 2005; Popik et al., 2002). The use of drugs that sequester cholesterol, such as methyl-ciclodextrins, has proven to disrupt lipid rafts and inhibit viral entry into the cell (Viard et al., 2002). Moreover, it has also been reported that the gp120 glycoprotein utilizes gangliosides present in lipid rafts as additional stabilizing factors for a successful entry to take place (Hammache et al., 1999; Nehete et al., 2002). Indeed, *in vitro* studies have shown that it is possible to prevent HIV-1 entry in the target cell both by inhibiting sphingolipid synthesis and by blocking specific sphingolipids with neutralizing antibodies (Harouse et al., 1991; Hug et al., 2000; Puri et al., 2004).

The entry of HIV-1 in the cell is not the only process in the infection cycle of the virus in which lipid rafts are important. In infected cells, several viral proteins are known to interact preferentially with raft microdomains in the cell membrane. The Gag and Gag-Pol precursors, for instance, possess myristoylation signals to ensure that these polyproteins are

directed to lipid rafts, the place where the assembly and release of new viral particles takes place (Lindwasser and Resh, 2001; Nguyen and Hildreth, 2000; Ono and Freed, 1999; Wang et al., 2000). In fact, studies have shown that the lipid composition of infectious HIV-1 virions is very similar to the one found in lipid rafts, and, additionally, cellular proteins that associate with lipid rafts have been detected in the envelope of HIV-1 viral particles (Aloia et al., 1993; Arthur et al., 1992).

In hope of further understanding the role and implication of lipid rafts in HIV-1 infection, we devised two different strategies interfering with these microdomains. On one hand, we specifically targeted a peptide fusion inhibitor to lipid rafts and assayed this construct in fusion and infection experiments. On the other hand, we replaced sphingomyelin by dihydrosphingomyelin at the membrane of target cells, by use of a synthetic ceramide analogue, and analysed this effect in fusion and infection assays.

Effect Of Membrane-Bound T20 In HIV-1 Infection

With the emergence of HIV-1 resistant strains to the antiretroviral drugs used in HAART (Martinez-Picado et al., 2000), there was a great need for the development of new drugs with different targets of inhibition. One attractive target was the entry of the virus in the cell, not only when the virus interacts with the cell receptors, but also when the gp41 subunits induce the fusion of the viral and cell membranes. For that purpose several C-peptides, derived from the HR2 region of gp41, were developed and tested in *in vitro* assays for inhibition potential (Chan and Kim, 1998; Wild et al., 1992; Wild et al., 1994). The mechanism of action of these C-peptides relies on the complementarity of their aminoacid residues to the HR1 region in gp41. Before the formation of the six-helix bundle, the gp41 subunits of the Env complex are found in a pre-fusion conformation with the HR1 regions exposed. At this stage, the C-peptides are able to recognize the complementary aminoacid residues in the HR1 region and attach to them, preventing the formation of the six-helix bundle and the consequent fusion of the membranes. Of the several C-peptides that showed promise in preventing HIV-1 entry in the cell, the T20 peptide was the most efficient and the first to be approved for clinical use (Kilby et al., 1998; Kliger et al., 2001; Wild et al., 1994).

The T20 peptide inserts in the membrane of target cells

Recent reports have indicated that the T20 peptide has the propensity to insert itself in the outer leaflet of liposomes (Veiga et al., 2004), and for that reason it has been suggested that the cell membrane might act as a reservoir of T20 molecules for when the virus approaches the cell. By incubating culture cells with soluble T20 for 24h we were able to confirm the incorporation of the peptide in the membrane of live cells, in a dose-dependent manner. The

fact that, *in vivo*, the membrane of target cells might harbour T20 peptides that are readily available to inhibit the entry of HIV-1 in the cell, lead us to design a strategy that utilizes membrane-bound T20 to prevent viral entry in the cell.

In 2001, Hildinger *et al* published a similar work using a T20-derived construct that is anchored to the membrane in an unspecific manner (Hildinger et al., 2001). They devised this strategy mainly as a response to the problems encountered with T20 administration in HIV-1 patients. In clinical use, T20 treatment is expensive and complicated, due to its lack of oral availability and the need for large amounts of the peptide to be delivered in order for an inhibitory effect to be produced. For these reasons the authors devised a strategy to express T20 in the membrane, as a gene therapy approach, in hope to surpass the drawbacks associated with T20 treatment. Our approach, however, differs from the one published by Hildinger *et al* in two main aspects: on one hand, we are anchoring T20 to the membrane specifically inside or outside lipid rafts, in hope to increase the local concentration of the peptide in sites of viral entry. On the other hand, by comparing the two different constructs, we expect to provide more evidence supporting the role of lipid rafts in HIV-1 infection.

Overexpression of T20 at the membrane is ubiquitous

We constructed two different T20 versions, T20LDL and T20GPI. In the case of the T20LDL construct, the transmembrane region of the low-density lipoprotein receptor (LDLR) anchors the construct to the membrane but specifically in a region outside lipid rafts, whereas T20GPI possesses a glycosylphosphatidylinositol (GPI) anchor that attaches the construct to the outer leaflet of lipid rafts. By isolating the DRM faction in flotation experiments, we were able to detect a specific band in the DRM lane in the case of the T20GPI stable cell line, indicating that this construct is present in raft microdomains. Furthermore, we did not detect any band in the DRM lane in the case of T20LDL cells, suggesting that this construct does not partition in lipid rafts. Unfortunatelly, an unspecific band at the same molecular weight as the T20 constructs masked the location of T20LDL in the solubilised protein fraction of the gradients. Nonetheless, we can conclude from these analyses T20GPI is present in lipid rafts, whereas T20LDL is not.

Concurring with this conclusion, we found that fluorescently-labeled antibodies induce clustering of the T20GPI peptide and the raft marker ganglioside GM1 by confocal microscopy. Unexpectedly, we found no difference in T20LDL or T20GPI colocalization with GM1; moreover, the levels of colocalization with GM1, i.e., presence in lipid rafts, are comparable to the ones found with a GFPGPI construct.

It is possible and likely that the high expression of the constructs in the membrane is responsible for the loss of specificity in localization. We could not rule out, however, that both constructs behave similarly and are not exclusively located at the desired site in the membrane, i.e. T20GPI in lipid rafts and T20LDL outside lipid rafts. Although the results from the DRM isolation seemed to indicate that the constructs partitioned in the expected site in the

membrane, the immunofluorescence experiments did not confirm this result.

Membrane-anchored T20 inhibits Env-mediated cell-cell fusion

Using the T20-expressing stable cell lines produced in HEK-293CD4 cells, we performed cell-cell fusion assays. The results indicate that both T20LDL and T20GPI inhibit Env-mediated cell-cell fusion equally well. Furthermore, this inhibition is comparable to the one achieved when the target cells are incubated with a low concentration of soluble T20 (between 0.5 and 1 μg/mL). Given the unavailability of a method to quantify the levels of T20 expressed in the membrane of stable cell lines, we cannot properly compare the inhibitory efficiency of soluble and membrane-anchored T20 peptides. Nevertheless, our results demonstrate that both T20GPI and T20LDL inhibition potential. However, this assay failed to show the difference between anchoring T20 in lipid rafts or outside them; as expected, both constructs inhibited fusion at similar levels, since they are undistinguishable at the level of membrane expression and localization.

The efficiency of membrane-anchored gp41-derived peptides in inhibiting cell-cell fusion was also reported by Egelhoher *et al*, in 2004 (Egelhofer et al., 2004). In this case, the authors used a modified version of the construct first reported in (Hildinger et al., 2001), with improved expression and antiviral activity, and replaced the T20 peptide with another C-peptide, C46. Their results confirmed that membrane-anchored C-peptides are efficient in inhibiting Env-mediated cell-cell fusion, as well as early steps of virus replication in single-round infection assays. From this work followed a more recent development, in which primary rhesus monkey CD4+ lymphocytes expressing membrane-anchored C-peptides against simian immunodeficiency virus (SIV) are protected from SIV entry (Zahn et al., 2008). Furthermore, the expression of modified C-peptides against human or simian immunodeficiency viruses (HIV-1, SIV or SHIV) in stable cell lines prevents the entry of HIV-1, SIVmac251 and SHIV89.6P. These results help support the notion that the expression of gp41-derived C-peptides at the membrane of target cells is a feasible mechanism that does not alter the antiviral activity that these peptides show when in solution.

Low expression of T20 in the membrane does not prevent infection by free HIV-1 viruses

In order to assess if membrane-anchored T20 is also able to block the entry of HIV-1 free viruses, we performed infection assays using replication-deficient HIV-1 pseudotypes. The target cells of infection were cells that express either T20LDL or T20GPI, but unfortunately we were not able to use the stable cell lines that express high levels of these constructs, since the infection levels were very low. To overcome this problem, we transiently transfected TZM-b1 cells with either T20LDL or T20GPI and infected them with HIV-1 replication-deficient viruses

pseudotyped with an ADA (R5 HIV-1 strain) or a VSVG envelope. However, the results obtained from the infection assays were different than the ones expected after the fusion assays. The TZM-b1 cells that expressed T20 at the membrane were permissive for ADA entry, regardless of whether they expressed T20LDL or T20GPI. Surprisingly, the entry of VSVG pseudotypes was inhibited in T20-expressing cells, especially in the case of T20GPI, where the infection levels were no higher than 30% when compared with control TZM-b1 cells. The susceptibility of ADA viruses to inhibition by soluble T20 was confirmed when control TZM-b1 cells were incubated with several concentrations of this peptide, whereas VSVG viruses infected the cells in spite of T20 presence in the medium.

The explanation for the fact that membrane-anchored T20 does not prevent the entry of ADA pseudotypes might rely on the T20 expression levels of the target cells. It is possible that only 23% of expression is not sufficient to produce an antiviral effect and prevent infection by HIV-1 viruses. The fact that we were not able to use the same stable cell lines used in the fusion assays hinders any direct comparison between the two experiments. It was reasonable to assume that either T20 construct would protect cells from infection by free HIV-1 viruses, taking into consideration not only the results from the fusion assays, but also the published works of Hildinger *et al* and Egelhofer *et al* (Egelhofer et al., 2004; Hildinger et al., 2001). However, the conclusion that can be drawn from the infection experiments is that low levels of T20 at the membrane of target cells are insufficient to protect the cells from infection by HIV-1 viruses.

HIV-1 pseudotypes produced in T20-expressing cells are more infectious than viruses produced in control cells

As was mentioned before, the assembly and release of new HIV-1 virions from the cell also takes place in lipid rafts (Nguyen and Hildreth, 2000). These microdomains serve as both portals of entry and exit from the cell, and the incorporation of cellular raft-associated proteins in the envelope of budding viruses is a common event (Arthur et al., 1992). We decided to investigate the infectivity of HIV-1 pseudotypes produced in cells that express T20LDL or T20GPI in the membrane. Our starting hypothesis was that, when the viruses exited the producing cell, they would incorporate T20 in the viral envelope, which would temper with the ability of those viruses to approach and gain entry into a target cell.

For this experiment we used not only the aforementioned ADA and VSVG envelopes, but also NL4.3 (X4 HIV-1 strain), and derivatives of these HIV-1 envelopes with reported mutations that confer resistance to T20-mediated inhibition: ADA 38E/42S, ADA 38A/42T and NL4.3 38E/42S. The first step was to test the infectivity of these viruses in cells treated with soluble T20 peptide. The results were surprising, because both wildtype NL4.3 and NL4.3 38E/42S pseudotypes were resistant to T20-mediated inhibition. In contrast, the 38E/42S combination in the ADA envelope rendered the virus resistant to inhibition by soluble T20, although the highest concentration of the T20 peptide was able to inhibit viral entry by 40%. Indeed, the reported IC_{50} for enfuvirtide using NL4.3 pseudotypes with this double mutation

is around 6.2 μg/mL (Sista et al., 2004), which would explain why 10 μg/mL of soluble T20, in our experiment, is able to inhibit viral entry to some degree. A similar explanation can be given for the ADA 38A/42T double mutant, because this virus was as susceptible to T20-mediated inhibition as wildtype ADA. The reason behind this result is that the published IC_{50} associated with this double mutation is only around 1.7 μg/mL (Sista et al., 2004), lower than the lowest concentration of T20 assayed. Therefore, no resistance to T20 was detected with the 38A/42T aminoacid combination. As expected, the control VSVG envelope infected cells regardless of T20 concentration.

We then produced HIV-1 replication-deficient viruses pseudotyped with these envelopes in stable cell lines that express high levels of T20LDL or T20GPI. Since the NL4.3 wildtype envelope resistant to T20-mediated inhibition, NL4.3 38E/42S viruses were not produced. Intriguingly, the results showed that both wildtype and T20-resistant ADA viruses produced in T20-expressing cells (ADA-T20) were several orders of magnitude more infectious than ADA viruses produced in HEK-293T control cells. The same was not observed for NL4.3 viruses produced in T20-expressing cells (NL4.3-T20), which infected target cells at roughly the same level as viruses produced in control cells. Once again, VSVG viruses showed no difference in infectivity whether they were produced in T20-expressing cells or not.

Our hypothesis that T20 incorporated in the envelope would decrease the infectivity of newly-formed virions by interfering with the fusion mechanism appears therefore wrong. However, in 2006 Melikyan *et al* published a similar strategy in which they induced the expression of membrane-anchored C46 in effector cells and used them in cell-cell fusion assays (Melikyan et al., 2006). They proposed that, due to the orientation of the C-peptide, it would not be able to engage the gp41 glycoprotein present in the same cellular membrane and inhibit the formation of the six-helix bundle. Their hypothesis was only half correct, because although the antiviral potency of these membrane-anchored C-peptides was indeed significantly reduced when compared to the same peptides expressed in target cells, fusion was still inhibited by 40%.

Another possibility is that the incorporation of T20 in the envelope of newly-formed virions alters the fluidity of the membrane. Several studies have demonstrated that the increase in membrane fluidity in viral particles renders them more infectious, probably due to an enhancement in multiple-site binding. Likewise, a decrease in membrane fluidity is responsible for the loss in infectivity of viral particles (Harada, 2005; Harada et al., 2004; Harada et al., 2005). However, if this were the case, then T20 used in soluble form would increase infection by HIV-1 viruses, since it has a tendency to incorporate itself in the lipid bilayer and it would increase fluidity of the target cell membrane, which has also been reported to enhance infection (Owada et al., 1998). The most likely explanation for the increased infectivity of HIV-1 virions that carry T20 is that the membrane-anchored peptide is able to enhance and stabilize the attachment of the virus to the target cell, allowing for the establishment of the necessary interactions between the viral and cellular proteins. It has been reported that the incorporation of host-derived major histocompatibility complex (MHC) class II glycoproteins in nascent HIV-1 viruses promotes infectivity by acting as additional adhesion molecules on the surface of the

target cell (Cantin et al., 1997; Tremblay et al., 1998). In our case, T20 would not be recognized by receptors present in the target cell, but it would allegedly insert itself in the outer leaflet of the cell membrane and anchor the virus to the site of entry. If this interpretation is correct, a major question is why the increased infectivity only happens for ADA-T20 but not for NL4.3-T20 viruses. Although a western-blot analysis to confirm the presence of T20 in the viral envelope is missing, it is expected that ADA and NL4.3 virions would be generated following the same pathway. The molecular explanation behind the increased infectivity of ADA-T20 viruses but not of NL4.3-T20 viruses in our experimental setup requires further experiments.

Effect Of The Inhibition Of Dihydroceramide Desaturase (DHCDase) In HIV-1 Infection

As was mentioned above, glycosphingolipids play an important part in HIV-1 entry in the cell. The implication of glycosphingolipids in the fusion process has been suggested by using PPMP, an inhibitor of the enzyme glycosyltransferase, which reduced the susceptibility of cells to infection and fusion by R5 and X4 HIV-1 viral strains (Hug et al., 2000; Puri et al., 2004). Furthermore, it has been found that the gangliosides GM3 and Gb3 are able to interact with the viral glycoprotein gp120, suggesting their implication in the entry process, possibly as stabilizing cofactors of the Env-CD4-coreceptor ternary complex (Hammache et al., 1999; Nehete et al., 2002). In addition, Harouse (Harouse et al., 1991) showed that antiglycosphingolipid antibodies are able to prevent HIV-1 infection *in vitro*.

These findings encouraged us to test the antiviral activity of a novel inhibitor of the ceramide biosynthetic pathway in HIV-1 infection. GT11 is a ceramide analogue that acts as a competitive inhibitor of the enzyme dihydroceramide desaturase (DHCDase). By inhibiting the enzyme, ceramide is not produced and its immediate precursor, dihydroceramide, predictably would accumulate in the cell. Consequently, saturated sphingolipids that lack a characteristic *trans* 4,5 double bond are synthesised, with different biophysical and structural properties than common unsaturated sphingolipids. GT11 was developed by Gemma Fabriàs laboratory at the IIQAB in Barcelona, and its inhibitory activity *in vitro* has been published (Triola et al., 2004; Triola et al., 2001); in our experiments we used two different GT11 compounds, GT11 C8 (with a fatty acid chain composed by 8 carbons) and hydrosoluble GT11pyr (a derivative of the original GT11 compound, with a pyrimidine ring).

We decided to study the implication of replacing unsaturated sphingolipids by saturated sphingolipids *in vivo*. Since lipid rafts are mainly composed by sphingolipids and cholesterol (Brown and London, 2000; Simons and Ikonen, 1997), it is reasonable to assume that this drug will affect lipid raft composition and function; therefore, we tested the effect of GT11 not only on basic raft-associated functions, but also on the ability to act as an antiviral agent against HIV-1.

GT11 inhibits both Env-mediated cell-cell fusion and entry of free HIV-1 viruses in a dose-dependent manner

As a proof-of-principle experiment, different concentrations of GT11 C8 were incubated with target cells in an Env-mediated cell-cell fusion assay. The results showed a dose-dependent decrease in the luciferase signal upon coculture of effector and target cells, indicative of the inhibition of cell-cell fusion caused by subtoxic doses of GT11 C8. Moreover, low concentrations of the compound were sufficient to induce an inhibitory effect (IC_{50} = 0.08 μM), further straightening the evidence that GT11 C8 is functional and able to inhibit the entry of HIV-1 in the cell.

Following the results stated above, we decided to test GT11pyr in infection experiments with replication-deficient HIV-1 pseudotypes. Two approaches were followed: in one case, high concentrations of GT11pyr were incubated with target cells for a short period of time, whereas in the other case low concentrations of the compound were used over a longer period of time. In the first approach, target cells incubated with GT11pyr reported a strong inhibition in viral entry (at least 60%) for viruses pseudotyped with HIV-1 specific envelopes (ADA and NL4.3), when compared with VSVG viruses, whose infection rate was unaffected by GT11pyr. This effect, moreover, was detected for all concentrations tested (ranging 15 μM upwards), indicating that the compound was strongly antiviral under the test conditions. Regarding the second approach, the inhibitory effect was more subtle due to the experimental conditions, but when the target cells were treated with 0.5 μM of GT11pyr for 84h the entry of ADA viruses was specifically inhibited (around 50%) compared to VSVG viruses. Therefore, a longer incubation period is necessary to inhibit viral entry when lower concentrations of the compound are used. The most likely explanation is that the synthesis of saturated sphingolipids, in the presence of GT11pyr, is cumulative, and lower concentrations of the compound need more time to induce the same level of replacement of unsaturated sphingolipids for saturated sphingolipids as higher concentrations do.

Subtoxic concentrations of GT11 do not affect T cell chemotaxis and activation

After the fusion and infection experiments, we are able to conclude that both GT11 C8 and GT11pyr possess antiviral activity against HIV-1 and are capable of inhibiting the entry of the virus in the cell. Presumably, this inhibitory effect derives from the change in lipid raft composition caused by the compounds. Given that lipid rafts are implicated in several cell processes (Fantini et al., 2002; Grassme et al., 2001; Inokuchi et al., 2000; Manes et al., 2003; Manes et al., 1999), it is important to verify that GT11 does not impede T cell-associated functions, such as chemotaxis and activation. Upon stimulation, whether it be an antigen-presenting cell (APC) or a chemokine, raft clustering is essential for the reorganization of receptors in the

membrane and the formation of supramolecular clusters that transduce the stimulation signal into a cascade of events inside the cell (Manes and Viola, 2006; Viola and Gupta, 2007). Since the use of other strategies that interfere with lipid rafts, namely cholesterol-sequestering drugs and sphingolipid depletion, have been known to impair certain cell functions (Hanada et al., 1995; Pike and Miller, 1998), it is crucial to assess if GT11 incubation induces the same kind of effect in target cells.

The results indicate that GT11 C8 or GT11pyr, at subtoxic concentrations, did not inhibit CXCL12-mediated chemotaxis of peripheral blood mononuclear cells (PBMCs) or Jurkat cells. In fact, a slight increase of the migration index was detected when Jurkat cells were incubated with GT11 C8 at 2 μM; less pronounced increases were registered for Jurkat cells incubated with 10 μM of GT11pyr, a concentration that affects cell growth, and PBMCs incubated with 2 and 5 μM of GT11pyr. Furthermore, non-stimulated PBMCs incubated with several concentrations of GT11pyr were able to produce interferon-γ (IFNγ) upon CD3- and CD28-mediated activation, an indication that GT11pyr treatment does not impair T cell activation mechanisms.

GT11 alters the lipid composition of lipid rafts but does not affect the localization of raft-associated proteins

Following the determination of GT11 antiviral activity in fusion and infection assays, and the confirmation that T cell-associated functions are intact, we next sought to verify if GT11 alters the lipid composition of lipid rafts by High Performance Liquid Chromatography coupled to time of fly mass spectrometry (HPLC-MS). The chromatograms showed that GT11pyr treatment induced a decrease in the amount of sphingomyelin (SM) in DRMs, associated with a corresponding increase in dihydrosphingomyelin (dhSM), the saturated version of sphingomyelin. Moreover, the dhSM/SM ratio was highest for 30 μM of GT11pyr, where a 30% replacement of SM for dhSM was detected. These results confirm that GT11pyr is able to inhibit DHCDase *in vivo*, and that such inhibition alters the lipid composition of lipid rafts.

Just as the fractions from DRM isolation were analysed by HPLC-MS, a western-blot to determine the presence of raft-associated proteins was also performed. In this case, we were interested in determining the membrane partitioning of the CD4 receptor in GT11pyr-incubated cells, since this receptor is used by HIV-1 to gain entry into the cell. The results from the western-blot showed us that CD4 partitioning to lipid rafts was unaltered when the cells were incubated with 30 μM of GT11pyr. This indicates that, although this concentration of GT11pyr is able to induce a substantial replacement of SM for dhSM in lipid rafts, the change in lipid composition does not affect the membrane localization of the CD4 receptor. In agreement with this idea, we found that GT11pyr did not change the colocalization of the CD4 receptor, the CXCR4 receptor or a GFPGPI construct with GM1, indicative of lipid rafts. An interesting observation is that the partition coefficient of CXCR4 in lipid rafts is lower than the one from the CD4 receptor, in non-stimulated conditions, as was reported before by Manes *et al* (Manes

et al., 2000).

Therefore, cell treatment with GT11pyr is able to alter the lipid composition of lipid rafts as theoretically proposed: sphingolipids no longer possess a ceramide backbone but instead a dihydroceramide one. This effect is expected because the inhibitor prevents the formation of ceramide in the cell, and the resulting sphingolipids mirror that inhibition. Strinkingly, this alteration is not sufficient to affect the localization of known raft-associated proteins, such as the CD4 receptor, the CXCR4 receptor and a GPI-anchored protein. This suggests that GT11pyr treatment is not as drastic as complete abolition of sphingolipid synthesis or the use of cholesterol-sequestering drugs, which are effective in preventing the entry of HIV-1 in the cell but also temper with other important cell functions (Hanada et al., 1995; Hug et al., 2000; Manes et al., 2000; Pike and Miller, 1998; Viard et al.).

Replacement of sphingomyelin for dihydrosphingomyelin increases membrane rigidity

The replacement of SM for dhSM in lipid rafts appears not to disrupt the formation of these microdomains, but alters their biophysical properties. The formation of negative curvature structures are a requisite for the occurrence of membrane fusion between the virus and the target cell (Peisajovich et al., 2000). Using different mixtures of dielaidoylphosphatidylethanolamine (DEPE) with SM, dhSM and cholesterol (Ch), we were able to assess the effect of dhSM on the lamellar-inverted hexagonal phase transition. The results suggest that replacing SM for dhSM does not difficult the formation of negative curvature structures, since there was no change in the transition temperatures of DEPE-dhSM bilayers when compared to DEPE-SM bilayers. However, when analysing the generalised polarisation (Gp) of Laurdan-labeled membranes with different mixtures of SM, dhSM and Ch, we detected an increase in Gp when dhSM is present, consistent with an increase in membrane rigidity. Moreover, when labelling giant unilamellar vesicles (GUVs) of PC:PE:SM:Ch (1:1:1:1) or PC:PE:dhSM:Ch (1:1:1:1) composition with the fluorescent probes DiI and NBD-Cer, we discovered that dhSM presence interfered with the incorporation of NBD-Cer. This indicates that the combination of dhSM and Ch originated a more rigid phase than SM and Ch in PC:PE:SM:Ch GUVs.

From these experiments we can conclude that the change in lipid composition caused by GT11 treatment leads to lipid rafts with increased rigidity. The antiviral activity detected with GT11 compounds might be explained by this increase in rigidity, since the correct functioning of the gp41 fusion peptide might be affected (Vincent et al., 2002), but we also questioned whether this alteration influenced other aspects of the entry process. In particular, we wondered whether the mobility of the receptors for HIV-1 entry in the cell was affected by GT11-mediated increase in membrane rigidity, since clustering of receptors at the membrane of the cell is a necessary step for the formation of the trimeric gp120-CD4-coreceptor complex (Manes et al., 2000; Nguyen et al., 2005; Popik et al., 2002). For example, cholesterol depletion has been shown to decrease the mobility of EGF receptors (Orr et al., 2005) and even of CCR5,

although it did not alter CD4 mobility (Steffens and Hope, 2004). However, the effect of increased membrane rigidity on receptor mobility has been less studied.

As an attempt to measure the mobility of the CD4 receptor in the membrane, we devised a single-particle tracking (SPT) experiment with GT11-treated cells. This technique consists on the analysis of the trajectories of single-fluorescent dots on the membrane of live cells, with a specific software (MatLab), and uses Total Internal Reflection Fluorescence Microscopy (TIRFM) to obtain the data for analysis. As opposed to Epifluorescence, in which the total fluorescence of a given sample when excited in a specific wavelenght is captured, TIRFM selectively illuminates and excites fluorophores in a restricted region of the specimen immediately adjacent to the glass-water interface. Consequently, only a thin section of the sample (around 100 nm) is visualised and background fluorescence from outside the focal plane is elliminated. Preliminary results obtained in Maria Garcia-Parajo laboratory (IBEC, Barcelona) with untreated HEK293CD4 cells showed that the mean square displacement (MSD) values for the CD4 trajectories analysed (n=39) were best adjusted by a curve that corresponds to confined diffusion, with a diffusion coefficient (D) of 0.059 $\mu m^2/s$. This indicates that the CD4 receptor, at the membrane of the tested control cells, exhibits a confined and not free diffusion, which might be indicative of the presence of the receptor in restricted environments. However, the analysis of the mobility of the CD4 receptor was not possible in GT11pyr-treated cells, due to slight alterations in the adhesivity of GT11pyr-treated cells which impeded the recording of image with enough quality for a confident analysis of the trajectories. We are currently studying alternative approaches to overcome these problems.

HIV-1 pseudotypes produced in GT11-treated cells are more infectious than viruses produced in control cells

Previous studies have determined that the lipid composition of HIV-1 viral particles is an important factor in their infectivity (Aloia et al., 1993). As such, our results with GT11 instigated us to produce replication-deficient HIV-1 pseudotypes (ADA) in cells treated with GT11pyr and analyse their ability to infect target cells. The results showed that, contrary to expectations, ADA viruses produced in GT11-treated cells (ADA-GT11) were twice more infectious than viruses produced in control conditions. Although a lipid analysis of the viral envelope was not performed, it is reasonable to assume that the viral particles exited the cells through lipid rafts and retained the lipid composition of these microdomains in their envelope, as previously reported. Therefore, we can infer that an increase in membrane rigidity favours viral entry in the target cell.

The reason for improved infectivity of ADA-GT11 pseudotypes is not immediately clear. We have mentioned before that membrane fluidity is important for HIV-1 entry in the cell, but Harada *et al* (Harada et al., 2005) have published that decreased membrane fluidity of viruses hampers their ability to infect target cells, while an increase in fluidity favours it. In fact, in 2005 and 2007 the same authors reported on two different antiviral agents, glycyrrhizin

and fattiviracin FV-8, which act as fluidity modulators and inhibit infection by several viruses including HIV-1 (Harada, 2005; Harada et al., 2007). They postulate that a decrease in membrane fluidity in cells treated with the compounds is responsible for inhibiting cell-cell fusion and infection by free viruses, similarly to GT11, although the mechanism of action was different: both glycyrrhizin and fattiviracin FV-8 are lipids that intercalate in the membrane of the cells and thus alter membrane fluidity. Furthermore, the authors refer that viruses treated with the compounds show a decrease in membrane fluidity that causes a reduction in viral infectivity, contrary to our results.

These discrepant results are difficult to explain, and probably suggest that the anti-retroviral effect of compounds that modify biophysical properties of the host and/or the viral membranes is complex and dependent of still unknown factors. Nonetheless, the results presented in this report are a very good starting point to think that these type of drugs can be added to the arsenal to fight against the HIV-1 pandemic.

CONCLUSIONS

1. The T20 peptide inserts in the membrane of live cells, in a dose-dependent manner.
2. The expression of high levels of membrane-anchored T20 inhibits Env-mediated cell-cell fusion. Transient expression of the membrane-targeted T20 peptides are unable to block entry of HIV-1 pseudotyped viruses.
3. ADA HIV-1 pseudotypes produced in T20-expressing cells are more infectious than HIV-1 pseudotypes produced in control cells. This probably implicates T20 as an additional stabilizing anchor in virus-cell interactions.
4. Targeting dihydroceramide desaturase (DHCDase) activity with GT11 and GT11 derivative compounds inhibits Env-mediated cell-cell fusion in a dose-dependent manner. Subtoxic doses of these compounds also specifically inhibit cell infection by HIV-1 pseudotyped viruses.
5. Inhibition of DHCDase activity *in vivo* produces a change in lipid rafts from unsaturated to saturated sphingolipids. The modification of lipid rafts at the lipid level does not result in an alteration of protein partitioning in detergent-resistant membranes, nor in an impairment of T cell chemotaxis and activation.
6. An increase in the dihydrosphingomyelin (dhSM) content of biological membranes enhances rigidity of liquid-ordered domains. This dhSM-induced increase in membrane rigidity may explain the ability of GT11 derivatives to inhibit HIV-1 entry.
7. ADA HIV-pseudotypes produced in GT11-treated cells are more infectious than HIV-1 pseudotypes produced in control cells.

Desarrollo de estrategias basadas en lipid rafts para inhibir la infección por VIH-1

INTRODUCCIÓN

El virus de la inmunodeficiencia humana (VIH) es el agente causante de una de las pandemias que ha causado más muertes en todo el mundo, el Síndrome de la Inmunodeficiencia Adquirida (SIDA) (Barre-Sinoussi et al., 1983; Hoffman, 2007; Levy et al., 1984). El VIH, un retrovirus perteneciente a la subfamilia *Lentivirinae*, tiene un genoma viral compuesto por dos moleculas de ARN dentro de una cápside ecosaédrica formada por proteínas virales. La capside está rodeada por una membrana lipídica derivada de la célula huésped, donde están anclados complejos de dos glicoproteínas virales (gp120 y gp41), importantes en el proceso de entrada del virus en la célula diana (Sierra et al., 2005; Turner and Summers, 1999). Dos especies de VIH han sido identificadas, VIH-1 y VIH-2, siendo VIH-1 la especie más virulenta y la causante de la mayoría de las muertes asociadas a este síndrome (Reeves and Doms, 2002).

El VIH-1 infecta esencialmente a linfocitos T y macrófagos (Dalgleish et al., 1984; Landau et al., 1988; Maddon et al., 1986). El ciclo de infección del virus comienza con la entrada en la célula diana, un proceso que requiere la interacción secuencial de la glicoproteína de la envuelta viral gp120 con el receptor CD4 en la membrana de la célula diana, y posteriormente con un receptor de quimioquinas, usualmente CCR5 o CXCR4. La formación del complejo de entrada gp120-CD4-coreceptor concluye con la fusión de la membrana viral con la membrana de la célula, y la posterior liberación de la cápside viral en el citoplasma de la célula huésped (Chan and Kim, 1998; Deng et al., 1996; Feng et al., 1996; Landau et al., 1988; Markosyan et al., 2003). Posteriormente el ARN viral es retrotranscripto en ADN de doble cadena, e insertado en el genoma celular. La transcripción y traducción del genoma viral en poliproteínas (Gag, Gag-Pol y Env) dará lugar a las proteínas esenciales para la formación de las nuevas partículas virales. Estas proteínas, junto con las dos copias del ARN viral se empaquetan y salen de la célula huésped a través de la membrana celular dando lugar a las nuevas partículas virales que, tras un proceso de maduración, estarán listas para iniciar un nuevo ciclo de infección (Bukrinskaya, 2004; Ganser-Pornillos et al., 2008; Gottlinger et al., 1989; Jouvenet et al., 2006; Nguyen and Hildreth, 2000).

Varios fármacos han sido desarrollados a lo largo de los años con el objetivo de inhibir algún paso en el proceso de infección del VIH-1 (De Clercq, 2002). Con la aparición de cepas virales resistentes a los fármacos existentes, ha surgido la necesidad de ampliar la terapia utilizada contra esta enfermedad, y desarrollar nuevos compuestos con distintas dianas de acción (Hughes et al., 2008; Martinez-Picado et al., 2000). Uno de esos nuevos fármacos es el péptido T20, también llamado enfuvirtide, que es capaz de inhibir la fusión de las membranas viral y celular a concentraciones muy bajas y, como consecuencia, la entrada del virus en la célula (Kilby et al., 1998; Wild et al., 1994). T20 es un péptido con una secuencia de aminoácidos derivada de la región HR2 de la glicoproteína gp41, y complementaria a la región HR1 de la misma glicoproteína, lo que permite su interacción con dicha región en un estadio previo a la fusión de las membranas. El resultado es la inhibición la formación de un complejo de seis

hélices α necesario para que las membranas viral y celular se fusionen (Chan and Kim, 1998; Wild et al., 1992; Wild et al., 1994). Este péptido ha surgido como una innovación en el campo de la lucha contra el SIDA, por ser el primer fármaco de la nueva clase antiviral de inhibidores de fusión.

Diversos estudios han demostrado que la infección por VIH-1 es un proceso dependiente de la integridad de unos dominios específicos de la membrana plasmática, denominados dominios de membrana tipo *lipid rafts* (Campbell et al., 2001). Los dominios de membrana *lipid rafts* son ensamblajes dinámicos de colesterol y esfingolípidos ricos en ácidos grasos saturados, que se localizan en la cara externa de la bicapa lipídica de la mayoría, si no de todas, las células eucariotas (Brown and London, 2000; Fridriksson et al., 1999; Simons and Ikonen, 1997). A pesar de su conformación ordenada, los *rafts* retienen una alta capacidad para difundir lateralmente en la membrana, aunque, a diferencia de los lípidos en dominios no-*rafts* que difunden como elementos individuales, los lípidos y proteínas incluidos en *rafts* difunden como un conjunto. Esta capacidad de movimiento permite, por un proceso de coalescencia, que proteínas inicialmente separadas en distintos *rafts* interaccionen entre sí (Harder and Simons, 1997; Simons and Ikonen, 1997). Todas estas características hacen que estos dominios de membrana tengan un papel activo en la regulación de diversos procesos celulares que abarcan desde la transducción de señales hasta la de entrada de patógenos intracelulares (Fantini et al., 2002; Grassme et al., 2001; Inokuchi et al., 2000; Manes et al., 2003; Manes et al., 1999). Diferentes estudios han demostrado que estos dominios son usados por el virus para regular la interacción tanto espacial como temporal, entre gp120, CD4 y CXCR4 o CCR5 y para servir de plataforma de entrada del virus, aunque el mecanismo aún no se conoce en su totalidad (Del Real et al., 2002; Manes et al., 2000; Nguyen et al., 2005; Popik et al., 2002). Estos dominios también son usados por el VIH-1 como anclaje para la salida de los nuevos virus (Aloia et al., 1993; Lindwasser and Resh, 2001; Nguyen and Hildreth, 2000; Ono and Freed, 1999).

OBJECTIVOS

1. Expresar quimeras de T20 ancladas a la capa externa de membranas celulares y analizar sus efectos en infección por VIH-1.

1.1 Generar líneas estables que expresen T20 anclado a la membrana, específicamente asociado o excluido de *lipid rafts*.

1.2 Analizar el efecto de T20 anclado a la membrana en fusiones célula-célula mediadas por las proteínas de la envuelta del VIH-1.

1.3 Analizar el efecto de T20 anclado a la membrana en infección por virus pseudotipados con envueltas de VIH-1.

1.4 Determinar la infectividad de virus pseudotipados con envueltas de VIH-1 producidos en células que expresan T20 en la membrana.

2. Inhibir la enzima dihidroceramida desaturasa (DHCDasa) y analizar su efecto en infección por VIH-1.

2.1 Determinar la toxicidad celular del inhibidor de DHCDasa GT11, y su derivado GT11pyr.

2.2 Analizar el efecto de GT11 en fusión célula-célula mediada por las proteínas de la envuelta del VIH-1.

2.3 Analizar el efecto de GT11 en infección por virus pseudotipados con envueltas del VIH-1.

2.4 Estudiar el efecto de GT11 en funciones celulares asociadas a linfocitos T.

2.5 Analizar la composición lipídica de *lipid rafts* en células tratadas con GT11.

2.6 Estudiar la localización de proteínas asociadas a *lipid rafts* en células tratadas con GT11.

2.7 Estudiar las propiedades biofísicas de membranas que contienen dihidroesfingomielina.

2.8 Determinar la infectividad de virus pseudotipados con envueltas del VIH-1 producidos en células tratadas con GT11.

RESULTADOS

1. Efecto del anclaje de T20 a la membrana en la infección por VIH-1

T20 es un péptido soluble que inhibe la infección por VIH-1 al bloquear la fusión entre la membrana celular y la viral (Kilby et al., 1998; Wild et al., 1994). Estudios previos han demostrado que el péptido T20 posee la capacidad de insertarse en la capa externa de liposomas, y que esa característica puede estar relacionada con la actividad antiviral demostrada *in vitro* (Veiga et al., 2004). Nuestros resultados han confirmado la propensión del péptido a insertarse en la membrana plasmática de células vivas, evidencia que nos ha incitado a generar células que expresen de forma estable el péptido en la membrana para favorecer su efecto antiviral. Generamos dos construcciones de T20 anclado a la membrana: una quimera de T20 asociada a dominios de membrana tipo *lipid rafts* (T20GPI) y una quimera excluida de estos dominios (T20LDL). Nuestro objetivo es aumentar la concentración del péptido en el sitio de entrada del virus, y proporcionar así nuevos datos que apoyen la importancia de los *lipid rafts* en la infección por VIH-1.

Inicialmente generamos líneas celulares estables que expresan altos niveles de T20LDL o T20GPI en la membrana. Comprobamos la localización de cada construcción en la membrana a través de gradientes de flotación en detergentes no iónicos y experimentos de inmunofluorescencia. Los resultados obtenidos de los gradientes de flotación indican que la construcción T20GPI está localizada en las fracciones correspondientes a membranas resistentes a la extracción por detergentes no iónicos, i. e. *lipid rafts*, mientras la construcción T20LDL no se encuentra en estos dominios. Desafortunadamente, la presencia de una banda inespecífica no ha permitido detectar T20LDL en la fracción correspondiente a proteínas solubilizadas. Los resultados de inmunofluorescencia, sin embargo, no han permitido confirmar este resultado ya que no se hallaron diferencias en la colocalización de cada construcción con el ganglíosido GM1 presente en los lipid rafts. Este resultado podría ser un efecto derivado de la sobreexpressión de T20 en la membrana, que origina una expresión ubicua de las construcciones y dificulta la distinción entre T20LDL y T20GPI.

Tras comprobar la expresión de ambas construcciones, el siguiente paso fue utilizar las líneas celulares estables para la expresión de T20 en ensayos de fusión célula-célula mediada por las proteínas de la envuelta del virus. Al poner en contacto células que expresan el complejo de la envuelta de VIH-1 en la membrana con células que sobreexpressan T20, se observó una reducción significativa en la fusión célula-célula tanto en el caso de T20LDL como en el caso de T20GPI. La eficiencia de T20 soluble para inhibir la fusión también ha sido comprobada. A diferencia del efecto de T20 que observamos en la fusión mediada por la envuelta viral, en ensayos de infección con virus deficientes en replicación pseudotipados con envueltas VIH-1 o control (VSVG), observamos que la expresión baja de T20 en la membrana de la célula diana no es suficiente para inhibir la entrada de virus que utilizan CCR5 como coreceptor de entrada

(cepa ADA), aunque el péptido soluble es eficaz en prevenir la infección por VIH-1 de un modo dependiente de la dosis usada.

Por ultimo, analisamos la susceptibilidad a la inhibición por T20 de virus deficientes en replicación pseudotipados con diferentes envueltas provenientes de VIH-1 y de VSVG, y descubrimos que los virus que utilizan CXCR4 como coreceptor de entrada (cepa NL4.3) son resistentes al péptido, mientras que virus ADA son susceptibles. Además, virus ADA con mutaciones descritas que confieren resistencia a T20 (38E/42S y 38A/42T) son parcialmente susceptibles a la inhibición por T20 soluble. Todos estos pseudotipos fueron además producidos en células que sobreexpresan T20 en la membrana, y se observó que los virus ADA son mucho más infecciosos cuando provienen de células con T20 en la membrana, comparando con los mismos virus producidos en células control, y hasta con virus NL4.3, en los que la presencia de T20 apenas aumenta la infectividad.

2. Efecto de la inhibición de dihidroceramida desaturasa (DHCDasa) en la infección por VIH-1

Existen muchas evidencias que apuntan a los esfingolípidos como factores esenciales para la entrada de VIH-1 en la célula, entre otras, se ha demostrado que la inhibición de la síntesis de esfingolípidos es capaz de prevenir la infección por VIH-1 (Hug et al., 2000; Puri et al., 2004). Por ese motivo hemos utilizado un compuesto, GT11, que inhibe la enzima dihidroceramida desaturasa (DHCDasa), responsable por la formación de ceramida a partir de dihidroceramida (Triola et al., 2004; Triola et al., 2001). Dos compuestos han sido usados en nuestros experimentos, por una parte GT11 C8, con una cadena larga de 8 carbonos, y GT11pyr, un derivado del GT11 original con una pirimidina.

Empezamos por analizar el efecto de ambos compuestos en la viabilidad de células T. Comprobamos que el tratamiento con GT11 C8 no afecta a la viabilidad de linfocitos aislados de sangre periférica (PBMCs), mientras que GT11pyr es tóxico en células Jurkat y PBMCs a concentraciones superiores a 5 μM. Para analizar la actividad antiviral de estos compuestos hicimos un ensayo de fusión célula-célula mediada por las proteínas de la envuelta viral utilizando células tratadas con distintas concentraciones de GT11 C8. Los resultados indican que GT11 C8 es capaz de inhibir la fusión célula-célula de un modo dependiente de la dosis, con una IC_{50} de 0.08 μM. Además, utilizando virus deficientes en replicación pseudotipados con envueltas VIH-1 comprobamos que GT11pyr es capaz de inhibir la infección de estos virus en células tratadas con el compuesto, de un modo dependiente de la dosis. Estos resultados indican que tanto GT11 C8 como GT11pyr son capaces de actuar como compuestos antivirales que previenen la infección por VIH-1.

Una vez establecida la eficacia de los compuestos para inhibir la entrada del virus en la célula, estudiamos el efecto de GT11 C8 y GT11pyr en funciones celulares asociadas a linfocitos T, como son la quimiotaxis y la activación a través del TCR. Los resultados indican que ninguno de los compuestos afecta negativamente a la quimiotaxis de células Jurkat y

PBMCs mediada por CXCL12, aunque observamos un incremento de la migración cuando tratamos las células con concentraciones altas. También detectamos que GT11pyr no afecta a la producción de IFNγ en PBMCs activados por CD3 y CD28. Asimismo, el tratamiento con GT11pyr no afecta a la localización de proteínas asociadas a *lipid rafts*, como son los receptores CD4 y CXCR4 y la proteína GFPGPI, a pesar de que el análisis de las fracciones de membrana resistentes a la extracción por detergentes no iónicos por HPLC-MS ha demostrado que hay un reemplazamiento de esfingomielina por dihidroesfingomielina cuando las células son tratadas con GT11pyr. Hemos demostrado que dicho reemplazamiento es capaz de aumentar la rigidez de la membrana, pudiendo explicar el efecto antiviral de los compuestos.

Por ultimo, observamos que virus deficientes en replicación pseudotipados con una envuelta ADA producidos en células tratadas con GT11pyr tenían una mayor infectividad que los mismos virus producidos en células control.

DISCUSIÓN

La entrada de VIH-1 en la célula diana es un proceso complejo que implica la interacción de la glicoproteína viral gp120 con los receptores celulares CD4 y CCR5/CXCR4, la formación del complejo trimérico gp120-CD4-coreceptor y finalmente la fusión de la membrana celular y viral mediada por la glicoproteína gp41 (Chan and Kim, 1998; Deng et al., 1996; Feng et al., 1996; Landau et al., 1988; Markosyan et al., 2003). La correcta formación del complejo de entrada del virus es dependiente tanto de la localización de estos receptores en dominios de membrana tipo *raft*, como de la capacidad de coalescencia de estos dominios (Del Real et al., 2002; Manes et al., 2000; Nguyen et al., 2005; Popik et al., 2002). No solo la entrada del virus en la célula es dependiente de *lipid rafts*, también la salida de los virus ocurre a través de estas regiones de membrana, con la incorporación de proteínas celulares asociadas a *rafts* en la envuelta de las nuevas partículas virales (Aloia et al., 1993; Arthur et al., 1992; Cantin et al., 1997; Lindwasser and Resh, 2001; Nguyen and Hildreth, 2000; Ono and Freed, 1999).

Con el objetivo de interferir de forma específica en la entrada del VIH-1 y determinar la importancia de la composición lipídica de la membrana de la célula diana para dicho proceso, llevamos a cabo dos abordajes, con la evidencia de que el virus utiliza los dominios de membrana tipos *rafts* como puerta de entrada. En primer lugar generamos péptidos con capacidad antiviral (T20) anclados de forma estable a la membrana de la célula diana, dirigiéndolos a dominios específicos de la misma. En un segundo abordaje alteramos químicamente la composición lipídica de la membrana celular, reemplazando esfingomielina por dihidroesfingomielina, y estudiamos su efecto en la infección por VIH-1.

El análisis de T20 comenzó confirmando que el péptido T20 es capaz de insertarse en la membrana de células vivas, como había sido descrito por otros autores en experimentos con liposomas (Veiga et al., 2004). Posteriormente, estudiando su efecto en la entrada del VIH-1, observamos que la expresión de T20 expresado en la membrana de células diana es capaz de prevenir la fusión célula-célula mediada por las proteína de la envuelta del virus. Sin embargo, no detectamos diferencias entre una versión de T20 anclada a los *lipid rafts* de otra excluida de estos dominios, posiblemente porque la sobreexpressión de estas construcciones las hace ubicuas en la membrana. Asimismo, podemos concluir que la baja expresión de T20 en la membrana no es suficiente para inhibir la entrada de virus ADA en las células, a pesar de que T20 en solución sí es efectivo en inhibir la infección por VIH-1. Este abordaje experimental es similar al trabajo publicado por Hildinger *et al* y Egelhofer *et al* (Egelhofer et al., 2004; Hildinger et al., 2001), en el cual los autores diseñan versiones de T20 y otros péptidos derivados de gp41 anclados a la membrana de células diana, determinando que estos péptidos poseen actividad antiviral no solo frente a VIH-1 sino también frente a SIV y SHIV. Nuestra estrategia va más allá, al diferenciar entre una versión de T20 asociada a *lipid rafts*, puerta de entrada del virus, o excluida de estos dominios. Asimismo comprobamos que la expresión en la membrana de péptidos derivados de gp41 no afecta a la capacidad antiviral que estos péptidos demuestran

en solución. Sin embargo, observamos que virus producidos en células que expresan T20 en la membrana plasmática son más infectivos. Este resultado contradice el trabajo publicado por Melikyan *et al* (Melikyan et al., 2006), donde se describe que T20 presente en la envuelta de los virus es capaz de inhibir la entrada en la célula diana. Es posible que, al igual que otras proteínas celulares existentes en la envuelta del virus que actúan como factores de anclaje del virus a la célula diana (Cantin et al., 1997; Tremblay et al., 1998), T20 pueda insertarse en la membrana de la célula diana y ayudar a estabilizar la interacción virus-célula.

En el segundo abordaje, determinamos que GT11 C8, un compuesto inhibidor de la enzima dihidroceramida desaturasa (DHCDasa) (Triola et al., 2004; Triola et al., 2001), previene la fusión célula-célula mediada por las proteínas de la envueltas de VIH-1 de un modo dependiente de la dosis. Confirmando este resultado, GT11pyr, un derivado hidrosoluble de GT11 C8, es capaz de inhibir la entrada de virus ADA y NL4.3 de un modo dependiente de la dosis. Estos resultados indican que ambos compuestos, a concentraciones subtóxicas, son efectivos como agentes antivirales. El tratamiento de células T con GT11 C8 y GT11pyr no afectan ni a su capacidad quimiotáctica mediada por CXCL12 ni a su activación vía TCR. Tampoco observamos ninguna alteración en la localización de proteínas asociadas a *lipid rafts*, incluyendo los receptores utilizados por VIH-1 para entrar en la célula. Sin embargo, estudios de HPLC-MS han demostrado que GT11pyr induce un reemplazamiento de esfingomielina por dihidroesfingomielina en los dominios de membrana tipo *raft* que, en consecuencia, conduce a un aumento en la rigidez de membrana. Estos datos pueden explicar la actividad antiviral de GT11, ya que un aumento en la rigidez de membrana afecta negativamente a la interacción de la glicoproteína gp41 con la célula, impidiendo la entrada del virus. Por ultimo, observamos que virus producidos en células tratadas con GT11pyr tienen aumentada su capacidad infectiva, resultado que contradice el trabajo publicado por Harada *et al* con otros compuestos (Harada, 2005; Harada et al., 2007). En dicho trabajo, y en otros (Harada et al., 2005), los autores describen que la disminución de la fluidez de la membrana viral inhibe la infección, mientras que un aumento la favorece. Nuestros datos indican que el efecto antiviral de drogas que modifican las propiedades biofísicas de las membranas, ya sea del virus o de la célula huésped, es complejo y dependiente de factores desconocidos. Sin embargo, los resultados mostrados en esta memoria nos incitan a pensar en compuestos moduladores de la fluidez de membranas como posibles fármacos a usar contra VIH-1.

CONCLUSIONES

1. El péptido T20 se inserta en la membrana de células vivas de un modo dependiente de la dosis.
2. La expresión de altos niveles de T20 anclado a la membrana inhibe la fusión célula-célula mediada por las proteínas de la envuelta del VIH-1. La expresión transitoria de T20 anclado a la membrana no bloquea la entrada del VIH-1.
3. Virus VIH-1 pseudotipados con la envuelta ADA producidos en células que expresan T20 en la membrana son más infecciosos que estos mismos virus producidos en células control. Este efecto podría deberse a que T20 actúa como estabilizador en la unión virus-célula.
4. La inhibición de la enzima dihidroceramida desaturasa (DHCDasa) por GT11 y compuestos derivados del mismo, bloquea la fusión célula-célula mediada por las proteínas de la envuelta del VIH-1 de un modo dependiente de la dosis. Concentraciones subtóxicas de estos compuestos inhiben la infección por VIH-1.
5. La inhibición de la enzima dihidroceramida desaturasa (DHCDasa) *in vivo* produce un reemplazamiento de esfingolípidos insaturados a saturados en los *lipid rafts*. Dicho reemplazamiento no afecta a la localización de proteínas asociadas a lipid rafts, ni a funciones esenciales de células T, como son la quimiotaxis y la activación vía TCR.
6. El incremento de dihidroesfingomielina en membranas biológicas aumenta la rigidez de la fase de membrana líquido-ordenada. Este aumento de rigidez podría explicar la capacidad de los derivados de GT11 para inhibir la entrada del VIH-1 en la célula.
7. Virus VIH-1 pseudotipados con la envuelta ADA producidos en células tratadas con GT11 son más infecciosos que los mismos virus producidos en células control.

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